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A PORPHYRIN PIGMENT FROM PHOTOSENSITIVE NON-CHLOROPHYLLOUS PLANT TISSUES[1]

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It has long been known that visible radiations exert direct control over the growth and development of plants by photoreactions other than those involved in photosynthesis. In recent years it has become clear, primarily through the determination of action spectra, that several of these apparently unrelated processes may have a common photoreceptor. For example, the action spectra for the inhibition of stem growth of albino barley and promotion of leaf growth of etiolated peas (1, 12), the photoperiodic control of floral initiation of cocklebur and other photoperiodically sensitive plants (2, 13, 14), and the photoreaction controlling lettuce seed germination (3, 6), appear to be similar, if not identical. Since light, to be photochemically effective, must first be absorbed, it follows that there must exist in all these tissues a pigment whose absorption spectrum should resemble the action spectrum obtained in vivo.

In all of the processes mentioned above, red light of wave length approximately 660 mμ is most effective, blue light is much less effective, and yellow and green light are almost completely ineffective. These facts led to the suggestion that the receptor pigment is an open-chain tetrapyrrole such as phycocyanin (1). However, such a pigment, though intensively sought after in higher plants, has never been found outside of the blue-green algae.

Recently, with the discovery that the red-light reaction controlling lettuce seed germination is immediately reversible by subsequently applied far-red irradiation at about 730 mμ (3), attention has been given to the possibility that the photoreceptor is actually a reversibly-reduced porphyrin. According to this interpretation, the relatively low promotive activity in the blue region of the spectrum would be due not to low absorption in this region, but rather to the low differential absorption between the promotive (660 mμ-absorbing) and inhibitory (730 mμ-absorbing) forms of the pigment. It seemed, therefore, that a new search for the photoreceptor pigment was in order. The most obvious plant material to use in such a search would be one showing a marked reaction to red light, but one relatively free of interfering pigments such as chlorophyll. The present work involves the isolation and characterization of a porphyrin, widely distributed in non-chlorophyllous photosensitive tissues, and of possible importance as the photoreceptor in several light sensitive processes.

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MATERIALS AND METHODS

In most of the experiments reported here, the initial extraction of pigment from the tissues was with peroxide-free diethyl ether (hereafter referred to as "ether"), petroleum ether, or acetone. The crude extracts were thoroughly washed with water (acetone extracts were first transferred to another solvent such as ether), followed by further solvent fractionation or by purification on sucrose or cellulose columns. Where seeds were to be extracted, they were usually ground in the dry state in a Wiley mill (20 to 40 mesh) and extracted with organic solvents. Tissues such as pea stems were usually lyophilized and then extracted, although some extracts were made of fresh tissue ground in a mortar. Although the pigment here described was present in all plant materials examined, the richest source was found to be the seeds of various *Poa* (bluegrass) species.

The best method found for the preparation of purified bluegrass seed pigment was as follows: The seeds were ground in a Wiley mill and extracted with ether until no further pigment was obtained. This extract was thoroughly washed with distilled water, and reduced to a small volume under vacuum. Residual water was removed by treatment with anhydrous sodium sulfate. The pigment was subsequently dissolved in hexane and placed on a cellulose column which had been prepared by pouring Whatman No. 1 filter paper powder into the column as an acetone slurry, and then thoroughly washed with hexane. A yellow pigment came off the column first, while two olive-green pigments and a yellow pigment remained on the column as long as only hexane was added. Then successively greater amounts of acetone were added to the hexane, and at a concentration of 15 to 20 % (V/V) acetone in hexane, the abundant olive-green lower band moved off the column, closely followed by the second yellow component. The slower-moving olive-green pigment, which

was not further investigated because it was present in very small quantities, could be removed from the column only with pure acetone. Since the absorption spectrum of this component closely resembled the first olive-green fraction, differences between these substances must reside in some relatively small feature of the molecule, such as a carboxyl group. Repetition of the above procedure yielded a highly purified preparation of the first olive-green pigment. Five kg of seed yielded about 20 mg of purified pigment. A reasonably pure preparation of the second green pigment can be obtained by extracting the crude ether extract with 15 to 20 % HCl, and transferring the pigment to ether by the addition of water.

of two 40 W daylight fluorescent tubes covered with red cellulose acetate film transmitting beyond 600 mμ. The far-red light source consisted of 300 W incandescent bulbs covered by Corning glass filters No. 587 (HR red-purple ultra). Incident energy was approximately 40 kiloerg/cm^2 × sec, as measured by a calibrated phototube radiation meter constructed by Dr. G. H. Bowen.

This latter instrument is essentially a rugged, portable, sensitive galvanometer. Current measurement is accomplished by means of a high gain DC amplifier, which, through negative feedback, equates the voltage produced by the input current flowing through a large resistance with the voltage produced

TABLE I

A COMPARISON OF PIGMENTS EXTRACTED FROM SEVERAL PLANT SOURCES

PIGMENT SOURCE	EFFECT OF RED LIGHT	REF.	EFFECT OF FAR-RED LIGHT	EXTRACTING SOLVENT	SOLVENT	PURITY	ABSORPTION MAXIMA (Mμ)
Lettuce seed var. Grand Rapids and var. Grand Rapids #1110	Promotes germination	(3)	Inhibits germination	Ether	Ether	Crude	405, 430, 600, 665
" " "	"		"	"	"	Purified	410, 505, 533, 610, 667.5
Kentucky bluegrass seed	Promotes germination	(20)	?	Ether	Ether	Crude	417, 445, 610, 665
" " "	"		"	"	"	Purified	410, 506, 534, 560, 610, 667.5
Etiolated pea stems	Inhibits elongation	(12)	Inhibits, antagonistic to red	Ether	Ether	Crude	410, 667.5
" " "	"		"	"	"	Purified	410, 535, 565, 610, 667.5
Albino barley leaves	Promotes elongation	(1)	Inhibits, antagonistic to red	Pet. ether	Pet. ether	Crude	422, 445, 670
Oat coleoptiles	Promotes elongation	..	Inhibits elongation	Acetone	Ether	Crude	400, 425, 665
Red top seed	Promotes germination	(20)	.....	Ether	Ether	Crude	610, 665
Timothy seed	Promotes germination	(20)	.....	Ether	Ether	Crude	610, 665
Tobacco seed	Promotes germination	(20)	.....	Ether	Ether	Crude	667.5
Oat seed	None		.....	Ether	Ether	Crude	665
" "	"		.....	"	"	Purified	405, 505, 530, 610, 655

Many different solvents were tested for their usefulness for paper chromatography. One of the best solvents for chlorophylls in ascending paper chromatograms was found to be 10 % acetone in hexane. The bluegrass pigment previously purified on cellulose columns moved with an Rf of 1.0 with such a solvent system. If 5 % acetone in hexane was used as the solvent, then the pigment moved with an Rf of 0.7. Ascending chromatograms were also used with lutidine saturated with water as the solvent (11), where an Rf of 1.0 was obtained.

For lettuce seed germination tests, 100 seeds were placed on 2 circles of moist Whatman No. 1 filter paper (4.25 cm) in Petri dishes 5.2 cm in diameter. The seeds were counted after 3 or 4 days germination at 21° C. The following light sources were used for irradiation treatments. The red light consisted

by the output current flowing through a small resistance. The current amplification is thus the ratio of the input to the output resistance, and is almost independent of the DC amplifier itself. Accurate readings can be obtained over the range 3×10^{-11} to 3×10^{-6} amperes.

The sensitive element is a type 926 vacuum phototube. This has a type S-3 spectral sensitivity (see Radio Corporation of America Tube Handbook) which is fairly uniform over the visible range. The relative response of this particular tube to narrow bands of wave length isolated from an incandescent source with Bausch and Lomb interference filters has been determined, calibration being made against a General Electric vacuum thermocouple (Type ZA-4).

All absorption spectra were obtained with a Beckman model DU spectrophotometer. The fluo-

TABLE II

A COMPARISON OF THE CHARACTERISTICS OF VARIOUS PORPHYRINS

PIGMENT	HCl NUMBER	PHASE TEST	ABSORPTION BANDS IN DIOXANE (mμ)						RATIOS OF ABSORPTION BANDS				
			I	II	III	IV	V	SORET	I/II	I/IV	I/V	SORET/I	REF.
Purified bluegrass seed pigment	15–17	–	667.5	610	ca. 560	535	505	410	6.32	5.38	4.01	2.51	....
Pheophytin a	28–29	+	667.5	609	559	535	500	...	6.16	4.98	4.26	...	(17, 5)
Pheophorbide a	15	+	662.5 *	606 *	556 *	531 *	503 *	412 *	6.45	4.99	1.67	...	(9, 5)
Methyl pheophorbide a	16	+	666	610	560	535	506	412	6.78	5.69	4.77	2.49	(15, 5)
Methyl pyropheophorbide a	15	–	667.5	609	559	535.5	507	...	6.71	5.51	4.39	...	(16, 5)
Pyropheophorbide a	12–13	..	Identical with methyl pyropheophorbide a						...	...	...	...	(5)

* In pyridine-ether.

rescence spectra were obtained with the aid of Dr. C. S. French of the Carnegie Institution of Washington, Stanford, California, on an instrument especially constructed for this purpose (7).

RESULTS

EXTRACTION OF THE PIGMENT: A survey was made of the pigment content of many plant tissues and organs, most of which were known to show some response to red light. The results of this survey are given in table I. All of the materials tested contained a pigment or pigments that absorb red light, and in most crude preparations the absorption maxima were at about 665 mμ. It was not usually possible to obtain much information about the blue light absorption of the crude extracts since they all contained considerable quantities of carotenoids or carotenoid-like pigments.

From the absorption spectra of the crude extracts, it would not be possible to distinguish between chlorophyll and related porphyrins. In the instances where further purification was done, the absorption was found not to be that of chlorophyll, but clearly that of some other pigment. In nearly all of these extracts, the chlorophyll content is extremely low or entirely lacking, while the other red-light absorbing pigment is present in significant amounts.

IDENTIFICATION OF THE PIGMENT: The primary means of identification of porphyrins is by their absorption spectra and their HCl number (5, 21). These are almost completely satisfactory when one is considering the separation of two unrelated porphyrins, or at least when their characteristics are dissimilar. For the final identification of many porphyrins some other method must be used since porphyrins differing only slightly often have identical spectral characteristics and HCl solubility. In these cases, degradation procedures are necessary.

The HCl number for the purified bluegrass pigment was found to lie between 15 and 17, and to be somewhat dependent on the impurities that were present (table II). The phase test (21) appeared to be negative. A comparison of the spectral characteristics of bluegrass pigment with those of several known related porphyrins is given in table II, and their struc-

TABLE III

ABSORPTION DATA FOR DERIVATIVES OF SEVERAL PORPHYRINS AND OF THE BLUEGRASS PIGMENT

	ABSORPTION BANDS (mμ)						RATIOS OF EXTINCTION BANDS			REF.
	I	II	III	IV	V	SORET	I/II	I/IV	I/V	
Methyl pyropheophorbide a oxime in dioxane	667.5	610	561	531	506	...	13.1	22.6	4.34	(18)
Bluegrass pigment oxime in ether	667.5	610	560	530	504	403	12.50	20.8	4.00	..
Purpurin 7 trimethyl ester (dioxane)	680	(636) weak	...	541	503	...	....	3.40	3.60	(18)
Bluegrass pigment + CH_3OH and HCl (ether)	667.5	610	...	532	502	...	7.17	5.80	3.74	..
							IV/II	IV/III	IV/V	
Pheoporphyrin a_5 dimethylester or monomethylester	...	634	583	562	521	...	8.55	1.27	1.71	(16)
Phylloerythrin monomethylester	...	634	581.5	557.5	517.5	...	6.93	1.52	1.52	(16)
Bluegrass pigment + HI in ether	...	ca. 635	ca. 582	558	518	...	6.28	1.44	1.24	..

tures are shown in figure 1. Since the apparent molar extinction coefficient was too low by a factor of 10 to permit comparison with known pigments, the ratios of the heights of the major absorption bands were compared. From these properties, it can be seen that the pigment most closely resembles methyl pyropheophorbide a, pyropheophorbide a, or possibly methyl pheophorbide a. From spectral characteristics, it is not possible to separate the methyl esters from the acids, although from the behavior of the pigment on lutidine-water paper chromatograms, it appears that the pigment has no free carboxyl group. Since there was no ash, it was concluded that no metal is associated with the porphyrin. The hemin derivative was prepared by adding freshly prepared ferrous acetate to a solution of the pigment (5), incubating at room temperature for 24 hours, then transferring the pigment to ether. This derivative gave an absorption maximum at 630 $m\mu$ in chloroform.

The oxime was formed by dissolving the bluegrass pigment in pyridine containing 15 % hydroxylamine hydrochloride. This was allowed to stand at room temperature for 7 days and the oxime was then extracted (4). A comparison was made of the spectral properties of the bluegrass pigment oxime and methyl pyropheophorbide a oxime (table III). Although this latter compound seems to agree very well with the unknown, it should be noted that no quantitative data on oximes is available for the other pheophorbides.

Reduction with HI would remove the two hydrogens from Ring IV and an ethyl group should be formed from the vinyl group on Ring I, if the respective groups were present in the molecule. If the unknown were methyl pheophorbide a (fig 1) (a carboxyl group on the cyclopentanone ring) then pheoporphyrin a_5 dimethyl ester would be formed. If, however, the unknown were methyl pyropheophorbide a (fig 1) (no carboxyl group on the cyclopentanone ring) then phylloerythrin would be formed (4). The results given in table III indicate that the latter was obtained.

A test used by Granick (8) to determine whether a carboxyl group is present on the cyclopentanone ring consists of reacting the pigment overnight at room temperature with methanol containing 30 % HCl. If a carboxyl group is present (as in pheophorbide a) then splitting of the ring occurs and purpurin 7 trimethylester is formed. However, the pigment appeared to be unaffected by this treatment (table III), and it was therefore concluded that the cyclopentanone ring is free of carboxyl groups.

It would seem that the unknown pigment most closely resembles methyl pyropheophorbide a. A comparison of the absorption spectra of the two compounds is shown in figure 2. The chemical tests indicate a vinyl group on ring I, a hydrogenated ring IV, and a cyclopentenone ring with no attached carboxyl group; there is no phytol group or metal in the ring.

There exists the possibility that the purified pigment represents an altered product of the native pigment. This is suggested by the fact that the crude pigment is not extracted by cold 15 % HCl from a crude ether extract of bluegrass seeds, but it is extracted with 30 % HCl; after purification, the pigment is extracted by 15 % HCl. This could indicate that the pigment molecule originally contained a phytol group, suggesting that its true nature is pheophytin or a closely related pigment. However, since all other characteristics point to the fact that the pigment is a pyropheophorbide, we prefer to attribute the altered HCl number to impurities such as fatty substances in the crude ether extract. Such impurities could make the pigment more soluble in the ether, and thus, the true HCl number would be obtained only after purification.

Physiological Role of the Pigment: One major question requiring investigation is the relation of this red-light-absorbing pigment to the red-light effects observed in vivo. Unfortunately, this question can at present be attacked only by indirect methods. However, such evidence as is available indicates that this pigment may serve as the in vivo photoreceptor.

Acetone pigment extracts were made from equal weights of several dark-seeded varieties of lettuce seeds showing different degrees of sensitivity to red light. It was found that the amount of pigment extracted by acetone, as determined by absorption at 665 $m\mu$ and 425 $m\mu$, was roughly proportional to the response of the seed to red light (table IV), with the exception of variety Bibb, which shows some light response but a very low pigment content. While this experiment is not conclusive, it does show that the highly-responsive lettuce seed varieties contain more red-light absorbing pigment than the non-light responsive seed. The correlation is not so close if the blue light absorption (425 $m\mu$) of the extracts is compared with the germination, but this latter absorption could be due to the variable amounts of other blue-light absorbing materials such as carotenoids present in the seed.

Because of the known far-red inhibition (730 $m\mu$) of lettuce seed germination, we must suppose the existence of some pigment in the seed which absorbs light in that region. However, in our experiments, no absorption peaks have been found in this region.

Data obtained by Hendricks (10) indicate that the red and far-red absorption by the intact seed may be due to the presence of two forms of a single pigment, these forms being interconvertible by appropriate wave lengths of light. To investigate this, three lots of lettuce seed var. Grand Rapids No. 1110 (each lot weighing 20 gm) were soaked in the dark for 4 hours. Two lots were then given far-red light for 30 minutes, one lot being maintained as an unirradiated control. One of the irradiated lots was subsequently treated with red light for 30 minutes, the other being placed directly in darkness. Each group of seed was homogenized in a Waring Blendor with 100 ml of cold acetone. The slurry was filtered and the residue again blended with 100 ml of acetone.

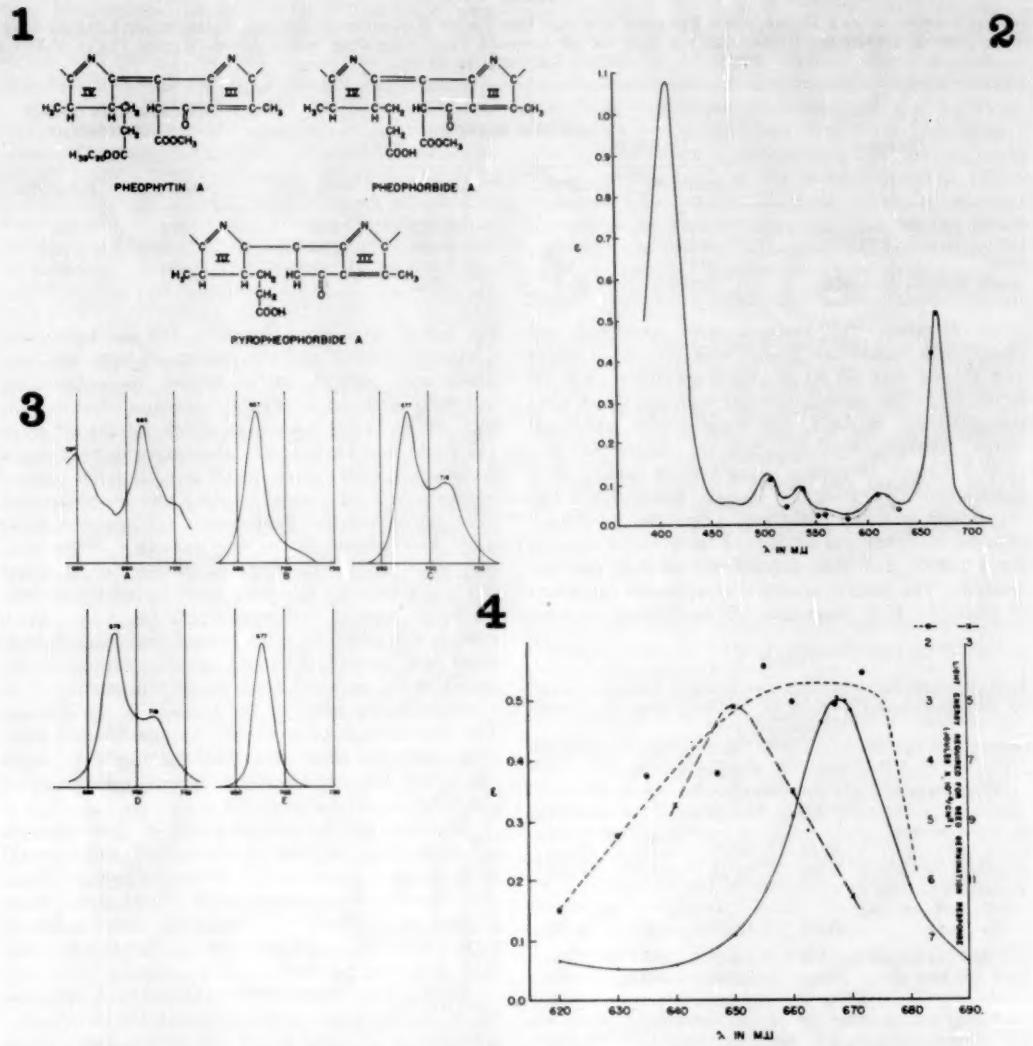

FIG. 1. Structures of several related porphyrins (5). Only pyrrole rings III and IV and adjacent atoms are shown. The methyl derivatives (e.g., methyl pheophorbide a) are identical with the parent compound (pheophorbide a) with the exception of a methyl substitution on the propionic acid group.

FIG. 2. A comparison of the absorption spectra in dioxane of purified bluegrass seed pigment (———) with methyl pyropheophorbide A (--○--) (Stern 16).

FIG. 3. Fluorescence spectra of plant organs and pigments. Exciting light source was wave length 436 mμ. Abscissae are wave lengths in mμ. Ordinates are relative fluorescent energies, all spectra being adjusted to the same height at the fluorescent band maxima. A. Dry ground Kentucky bluegrass seed; B. Ground bluegrass seed with acetone added; C. Purified bluegrass seed pigment in ether; D. Pheophytin a prepared from chlorophyll a, in ether; E. Albino barley leaf—exposed to light as little as possible.

FIG. 4. A comparison of the lettuce seed germination action spectra with the absorption spectrum of extracted seed pigment. A. (———) Absorption spectrum of the purified pigment in dioxane. B. (—×—) Lettuce seed germination action spectrum. Monochromator as the light source. Curve for promotion to 85% germination. C. (--○--) Lettuce seed germination action spectrum of Borthwick et al (3). Curve for promotion to 50% germination.

TABLE IV

A COMPARISON OF THE EXTRACTABLE PIGMENT AND THE RED LIGHT RESPONSE OF SEVERAL VARIETIES OF LETTUCE SEED. THE OPTICAL DENSITIES GIVEN ARE FOR 30.0 GM OF LETTUCE SEED EXTRACTED WITH ACETONE, THE FINAL VOLUME BEING ADJUSTED TO 50 ML

VARIETY	OPTICAL DENSITIES				% PROMOTION OF SEED GERMINATION BY RED LIGHT	
	665 mμ		425 mμ			
	ABSOLUTE	RELATIVE	ABSOLUTE	RELATIVE	ABSOLUTE	RELATIVE
Grand Rapids	0.071	100	0.522	100	672	100
Grand Rapids #1110	0.060	84.5	0.490	93.9	382	56.9
Bibb	0.012	16.9	0.218	41.7	104	15.5
Black Seeded Simpson	0.019	25.8	0.267	51.2	0	0

After filtration, the extracts were combined and placed in a separatory funnel with 200 ml of water and shaken with 50 ml of petroleum ether (B.P. 30 to 60° C). The petroleum ether solution, which then contained the pigment, was washed with additional water, centrifuged to separate the layers and the optical density at various wave lengths subsequently determined in a Beckman spectrophotometer. The experiment was also performed where the seeds were exposed to either red or far-red light (after soaking for 4 hours) and then ground immediately and extracted. The results of these experiments are given in table V. It is clear that the amount of pigment

TABLE V

INFLUENCE OF RED (660 mμ) AND FAR-RED (730 mμ) LIGHT ON THE PIGMENT CONTENT OF LETTUCE SEED VAR. GRAND RAPIDS NO. 1110

TREATMENT	OPTICAL DENSITY			
	667.5 mμ	610 mμ	535 mμ	412 mμ
Dark only	0.118	0.027	0.037	0.510
Far-red (30 min.)	0.106	0.022	0.033	0.507
Far-red (30 min.) followed by red (30 min.)	0.057	0.012	0.018	0.352
Far-red (45 min.)*	0.149	0.047	0.057	0.780
Red (30 min.)* ..	0.088	0.030	0.043	0.600

* The optical densities of the treatments below are not comparable with the ones above since different starting weights of seeds were used.

absorbing at 667.5 mμ is not greatly affected by far-red, but is decreased to about one half through irradiation by red light. The predicted simultaneous appearance of a peak at 730 mμ could not be detected.

FLUORESCENCE STUDIES ON THE PIGMENT: Several unsuccessful attempts have been made to detect absorption of red light by intact, chlorophyll-free plant tissues sensitive to red light (1). Because fluorescence can be detected at lower concentrations of pigment than are required for the detection of absorption, the fluorescence spectra of several non-chlorophyllous light-sensitive tissues were investigated. Using the apparatus designed by French (7), the tissues were irradiated with 436 mμ light from a monochromator, and the fluorescent light was analyzed and plotted by a second monochromator equipped with an automatic recording device. Intact albino barley leaves, in which Borthwick et al (1) could find no red-light absorption, gave a slight fluorescence with a peak at 677 mμ (fig 3 E). Intact lettuce seed (var. Grand Rapids) and bluegrass seed gave no detectable fluorescence. When the same seeds were ground in the dry state in a Wiley mill, they gave slight indications of fluorescence at about 665 mμ, Kentucky bluegrass seeds giving a measurable fluorescence at that wave length (fig 3 A). When acetone was added to these ground seeds, this fluorescence was greatly enhanced, possibly owing to removal of the pigment from its protein carrier or to a concentrating effect of the pigment in the solvent. The fluorescence peak of the acetone-treated bluegrass seed was then about 667 mμ (fig 3 B), while the lettuce seed with acetone showed fluorescence at about 670 mμ and also at 633 mμ.

The observed fluorescence peaks of these seeds do not correspond to those of chlorophyll, which would be expected to occur at 677–685 and 725 mμ. Thus, this observed fluorescence could indeed result from a pigment other than chlorophyll, which pigment may act as the photoreceptor in the various red-light requiring processes.

ACTION SPECTRUM DETERMINATIONS: A comparison of the published action spectrum for lettuce seed germination (3) and of our absorption spectrum of the pigment extracted from lettuce seeds reveals certain discrepancies between the two. Most striking is the fact that while the action spectrum has a very flat maximum from about 640 to 670 mμ, the pigment has a sharp absorption maximum in ether solution at 667.5 mμ. An attempt was therefore made to show up possible fine detail in the action spectrum for the germination of lettuce seed. Two independent methods were used:

1. An apparatus was utilized involving incandescent light passed through Corning glass absorption and Bausch and Lomb interference filters. These filter pairs gave sharp transmission peaks (10 mμ half-band width) at 20 mμ intervals. Wave length stations were at 620, 640, 660, and 680 mμ, and the incident energy at all stations was equalized by ad-

justment of variable rheostats mounted in series with each incandescent source. Lots of 100 seeds were then exposed to varying energies at each wave length, and energy required to increase germination to a standard percentage calculated.

2. A monochromator from a Beckman model DU spectrophotometer was used as a light source. Lenses and a diaphragm were so arranged as to give enough uniform light to irradiate 100 lettuce seeds in a small Petri dish. For the irradiation at different wave lengths, the same slit width was used, incident energy being measured on a calibrated phototube light meter. Energy was varied by varying the length of time of exposure, and a curve was thus obtained relating percentage germination to incident energy at each wave length. From such curves, we again calculated the relative energies required to increase germination to some given percentage.

The action spectra obtained by these two different methods agreed very well, showing a fairly sharp peak at about 650 mμ. Such a peak also seems to be present in the action spectrum of Borthwick et al (3) though the curve which they draw does not emphasize this point. The action spectrum obtained with the monochromator is compared with the absorption spectrum of the extracted pigment in figure 4. The shape of the curve approximates what would be expected from the sharp maximum obtained with the extracted pigment, assuming only that the attachment of the pigment to protein in vivo causes a shift in its absorption spectrum of about 17 mμ toward the short wave length end.

Discussion

To our knowledge, there are no previous reports of the occurrence of this general type of pigment in the free form in any biological material. The fact that it can be found so readily in tissues which are markedly light sensitive implies at once its possible role as a photoreceptor.

As discussed previously, the fact that the apparent HCl number of the purified pigment differs from that of the crude pigment may indicate that the pigment is altered during extraction and purification. However, this discrepancy could as well be attributed to removal of substances such as fats, which may mask the true HCl number.

The evidence here presented on the biological role of the pigment is admittedly inconclusive. It would be desirable to show that irradiation with 660 mμ leads to disappearance of the red peak and simultaneous appearance of a peak at about 730 mμ; irradiation with 730 mμ should then produce the reverse effect. If such shifts result from reversible oxidation-reduction of porphyrins, then they probably occur only when the porphyrins are linked to specific proteins. This means that the reaction must probably be looked for in vivo rather than in vitro, despite the fact that our in vivo experiments were unsuccessful in turning up a peak in the far-red. This failure could of course be due to the lability of the presumed reduction product having a peak in the far-red.

It should be noted that the fluorescence spectrum of the purified bluegrass pigment shows a peak at 677 mμ and another at 716 mμ. The pigment would here seem to have energy levels capable of satisfying the known action spectrum data. The discrepancy between the action spectrum peak (ca. 650 mμ) and the absorption peak of the isolated pigment (667.5 mμ) could be attributed to alteration of spectral absorption as a consequence of attachment of the pigment to a protein. Thus, all the data from absorption spectra, fluorescence spectra and action spectra in the red region can be harmonized reasonably well.

One major obstacle in accepting this pigment as the photoreceptor is the fact that it has a marked absorption in the blue, while the action spectrum shows relatively low effectiveness in the blue. Although this can be rationalized in terms of differential absorption of blue light by two pigment forms having opposite biological effects, our failure to obtain the predicted peak of the reduced pigment in the far-red fails to substantiate this type of interpretation.

Summary

An olive-green pigment has been found in low concentrations in several non-chlorophyllous plant organs that respond to red light.

The extraction and purification of this pigment from bluegrass seed is described, and absorption and fluorescence spectra are given for the purified pigment. Major absorption maxima occur at 410 and 667.5 mμ and minor maxima at 505, 534 and 610 mμ. A fluorescence maximum of the pigment in vivo occurs at 665 mμ; in the purified pigment this maximum is shifted to 677 mμ. Chemical and physical tests performed on the pigment indicate that it is a metal-free porphyrin, similar to or identical with methyl pyropheophorbide a.

Several lines of indirect evidence indicate that the pigment may be involved as photoreceptor in the various red-light sensitive processes:

a. Irradiation of lettuce seeds with red-light results in a decrease in extractable pigment with an absorption maximum at 667.5 mμ.

b. The light sensitivity of several varieties of lettuce seeds correlates with the quantity of pigment extractable from such seeds.

c. Action spectra for the promotion of germination of lettuce seeds by red light have been determined both by a monochromator device and by an interference filter device. A fairly sharp peak obtained in the region of 650 mμ could be attributed to photoreception by the pigment here described.

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POSITION OF C^{14} IN ALCOHOL AND CARBON DIOXIDE FORMED FROM LABELED GLUCOSE BY CORN ROOT TIPS[1, 2]

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There are good reasons for supposing that the classical pathway of glycolysis (Embden-Meyerhof-Parnas, or E.M.P., sequence) plays an important role in the respiratory breakdown of glucose in higher plants. The evidence has come not only from experiments on cell-free systems capable of fermenting glucose to alcohol and CO_2 but also from work with intact tissues respiring in air and nitrogen (reviewed by Thomas, 17). The close analogy between fermentations induced by yeast and by higher plant tissues, and the recent isolation of some of the enzymes responsible for glycolysis from green plants (16) have justified the belief that the glycolytic mechanisms involved in the two cases are the same.

Recently, however, evidence has accumulated that alcohol and CO_2 may be formed during bacterial dissimilation of glucose by pathways (7, 10) other than the classical glycolysis, and, moreover, enzymes responsible for the reactions of one of these alternate pathways (the hexose monophosphate shunt or oxidative pathway) have been characterized in higher plant tissues (1, 2, 9). It was thus of interest to re-examine glucose breakdown in a plant tissue using methods which would reveal whether a mechanism other than the E.M.P. sequence was contributing to alcohol production and thus to alcohol precursors such as pyruvic acid.

The technique used was one which has been ap-

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plied successfully to determine the pathway of glucose dissimilation in yeast (12), fungi (8), and microorganisms (6, 7, 10). Glucose labeled with C^{14} in specific positions is metabolized by the organism, and the end products or intermediates are isolated and degraded in order to determine the location of the glucose carbon atoms. Thus if glucose breakdown was occurring by the E.M.P. sequence in the organisms being tested, the position of the tracer in the end product alcohol could be predicted, and if this sequence was operating to the exclusion of the direct oxidation pathway the predictions would be fulfilled. Clearly, if the locations of the glucose carbon atoms in the alcohol were different from those predicted, and in accordance with what is known about the reactions of the direct oxidation pathway (11), this would constitute evidence of its operation.

The possible occurrence of these pathways in young corn roots has now been studied in this way. The following substrates were used: a) glucose-1-C^{14} in a N_2 atmosphere and b) glucose-1-C^{14}, glucose-2-C^{14} and glucose-3,4-C^{14} in the presence of O_2 with sodium arsenite (which restricts the oxidation of pyruvate and diverts it to alcohol and CO_2). Following dissimilation of glucose, the two carbons of alcohol and that of the respired CO_2 were analyzed individually for radioactivity content to determine the distribution of isotopic carbon. The results are the subject of the present paper.

Materials and Methods

Plant Materials: Corn hybrid Wf 9×38-11 was soaked in water for 24 hours, drained and allowed to germinate at 25° C. When the primary roots had reached a length of 3 to 4 cm the tips (1 to 2 cm) were cut off, washed in distilled water and used without further treatment. Twenty tips per flask were usually used in the manometric experiments but in the gas stream experiments 2 to 4 gm of tissue was used as a sample.

Manometric Experiments: All experiments were carried out at 25° C using the conventional Warburg respirometer. The main compartment of the Warburg vessel contained the root tips, 1 ml of 0.03 M phosphate buffer at pH 5.0 and water to make a volume of 2.0 ml. The side arm carried the sugar sample (10 to 60 micromoles) while the center well contained 0.2 ml of 20 % KOH. In the anaerobic experiments the vessels were filled with prepurified N_2 and equilibrated before closing and adding the glucose from the side arms. At the end of the manometric experiments the KOH was removed from the center well and transferred to a flask filled with N_2. The center well was washed three times with CO_2-free water and the washings included with the KOH. The CO_2 was liberated by acid and passed into a known amount of 0.3 M $Ba(OH)_2$ and its amount determined by back titration with 0.1 N HCl. Usually the amount of CO_2 produced during the experiment was large enough to make the addition of carrier CO_2 unnecessary: i.e., sufficient $BaCO_3$ (about 20 mg) was produced to give a layer of near infinite thickness when it was transferred to a sintered glass disc for determination of radioactivity.

Gas Stream Experiments: The tissue was suspended in 8 ml of 0.03 M phosphate buffer, pH 5.0, in a 30 ml cylindrical, sintered glass funnel of medium porosity. 60 to 120 micromoles of glucose were added and a stream of CO_2-free air or prepurified N_2 was passed into the base of the funnel and through the solution bathing the roots. The gas stream was then passed through two absorption tubes in which the respired CO_2 was collected in 0.3 M $Ba(OH)_2$. In some of the experiments with arsenite the outgoing gas stream was first passed through 2 % sodium bisulfite to absorb acetaldehyde and then through half-saturated potassium permanganate before it entered the CO_2 absorption tubes. The amount of CO_2 produced was determined by titrating the excess $Ba(OH)_2$ with 0.1 N HCl and the $BaCO_3$ was transferred to sintered glass discs for determination of its radioactivity.

Alcohol Degradation: At the end of the experimental run an aliquot of the solution bathing the roots was removed and the amount of alcohol determined by means of yeast alcohol dehydrogenase (5). The rest of the solution was made alkaline to phenol red with 1 N KOH and the alcohol distilled with carrier alcohol. It was oxidized to acetic acid by heating at 95° C for 2 hours with 0.5 gm of $K_2Cr_2O_7$ in 4 N H_2SO_4. The acetic acid was distilled, neutralized with 0.1 N NaOH and evaporated to dryness. The sodium acetate was then degraded by the method of Phares (13).

Measurements of Radioactivity: All C^{14} samples were assayed as barium carbonate, using a methane-flow beta proportional counter (14).

Labeled Glucose: The glucose-1-C^{14} and glucose-2-C^{14} were kindly supplied by Dr. H. Isbell of the National Bureau of Standards. The glucose-3, 4-C^{14} was prepared from rat liver glycogen (18).

Results and Discussion

Some preliminary experiments were carried out in which 20 to 40 micromoles of uniformly labeled glucose (c. 20×10^{-9} curies) were applied to corn roots respiring in N_2 or in air. These established the important fact that, even though the glucose might not induce an increase in the high endogenous respiration rate, as judged from the measurements of gas exchange, nevertheless the exogenous glucose was utilized, as shown by the progressive release of $C^{14}O_2$. The aim was to supply to the respiring material a small amount of glucose (to limit dilution of radioactive material) and yet to provide an amount sufficient to give easily measurable amounts of radioactivity in the respiratory products.

Anaerobic Experiments with Corn Roots: Two types of experiments were carried out (table I): one of the gas stream type (experiment 1) and the other in Warburg vessels (experiment 2). In both experiments, it is clear that of the radioactivity recovered in the products, almost all of the aldehyde

TABLE I

THE LOCATION OF C^{14} IN THE CO_2 AND ETHANOL PRODUCED ANAEROBICALLY BY CORN ROOT TIPS * FROM GLUCOSE-1-C^{14}

EXPERIMENT	PRODUCT	SPECIFIC ACTIVITY **	TOTAL ACTIVITY	% OF TOTAL
		mμ c/mg C	*mμ c*	
I. Gas stream 4.5 hrs.	CO_2	0.10	0.34	4.6
	CH_3OH of alcohol	0.03	0.24	3.3
	CH_3 of alcohol	0.99	6.77	92.1
II. Manometric 4.25 hrs.	CO_2	0.16	0.21	7.6
	CH_3OH of alcohol	0	0	0
	CH_3 of alcohol	1.04	2.55	92.4

* In experiment I, 4-gm roots were suspended in 10 ml solution containing 120 micromoles glucose (specific activity 7.5 mμ c/mg C). 13.5 mg respired CO_2 was collected and no carrier was added. From the alcohol/CO_2 ratio of 0.92 observed previously with this tissue (4) it was calculated that 12.4 mg alcohol had been produced and 22.1 mg inactive alcohol was added as carrier before distillation.

In experiment II, a total of 160 root tips respired in 8 ml solution containing 60 micromoles glucose (specific activity 7.5 mμ c/mg C). 4.82 mg CO_2 were produced and Na_2CO_3 equivalent to 8.1 mg CO_2 was added as carrier. 22.8 mg inactive alcohol was added to the calculated 4.43 mg alcohol produced before distillation.

All figures are corrected for dilution by carrier.

** Specific activity expressed as millimicrocuries per milligram of carbon.

carbon of glucose was in the methyl group of alcohol; the carbinol group was essentially unlabeled and a trace of the isotope appeared in the CO_2. Since the location of the C^{14} was such as might have been expected from the E.M.P. scheme (12), it would appear that under anaerobic conditions, this was the only pathway of glucose breakdown being used by the corn roots.

AEROBIC EXPERIMENTS WITH CORN ROOTS AND ARSENITE: From Warburg experiments designed to find a concentration of arsenite which would prevent O_2 uptake and induce accumulation of pyruvate it soon became apparent that the corn tissues reacted differently from those of other species to which this method has been successfully applied in the past (6). The results are shown in figure 1. It will be seen that although O_2 uptake is strikingly reduced at concentrations as low as 10^{-3} M, the addition of more arsenite does not lead to a further steep decline in the curve, and at the highest concentrations used the residual O_2 uptake was still maintained at about 40 % of the control level. Even more significant, however, was the effect on R.Q. and CO_2 release. Whereas the control roots respired with an R.Q. of 1.08, those in the presence of increasing amounts of arsenite showed progressively higher R.Q.'s which reached a maximum of about 2.3. A calculation of the excess CO_2 over that which would have been expected from the O_2 uptake values if the R.Q. had remained at the control level yields curve "F". It is quite evident that excess CO_2 appears only when concentrations inhibitory to O_2 uptake are applied, and, as the O_2 uptake curve declines, so the "F" curve rises. Such effects of an inhibitor are reminiscent of others (3, 4) in which it has been shown that the excess CO_2 is due to induced aerobic fermentation. It has now been shown, in fact, that a similar explanation holds for the arsenite curves; alcohol and acetaldehyde accumulate as a result of arsenite poisoning. Aerobic fermentation is known to be induced by a variety of agents which for one reason or another might be expected to lead to an increased internal concentration of pyruvate, which does not accumulate in large amounts but is diverted towards alcohol through the action of carboxylase. Arsenite, it now appears, acts in a similar manner to induce aerobic fermentation; the accumulation of pyruvate, which results from the slowing down of its utilization by arsenite in other species, finds, as its counterpart in the corn root, the accumulation of alcohol and acetaldehyde.

Advantage was taken of this effect of arsenite in another group of experiments with differently labeled glucose samples (glucose-1-C^{14}, glucose-2-C^{14}, and glucose-3,4-C^{14}). The procedure was to collect the alcohol produced in air in the presence of M/200 arsenite and to determine the distribution of C^{14}. All of these experiments were of the gas stream type, and acetaldehyde was collected in some of these, only to confirm that it was produced (15); it was not degraded. In a 4 hour run with inactive glucose plus M/200 arsenite in which the three respiratory products were measured the following values were obtained: CO_2: 3.10 mg, acetaldehyde: 0.41 mg, alcohol: 1.88 mg. Since a considerable amount of aerobic respiration (as judged from O_2 uptake values in figure 1) persisted at this arsenite level, it was not surprising to find that activity appeared in the respired CO_2 from each of the labeled-glucose samples; as pointed out above, attention was focussed on the alcohol. Table II shows the results of the degrada-

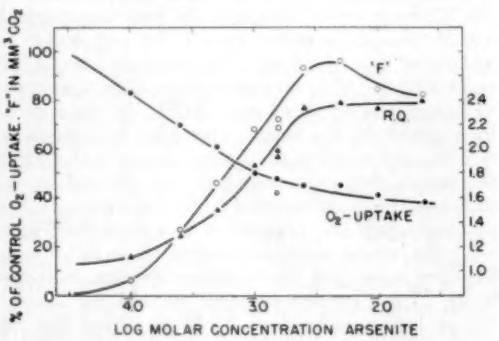

FIG. 1. The effects of graded concentrations of sodium arsenite on O_2-uptake and R.Q. of corn root tips. The "F" curve is calculated from the R.Q. and the O_2-uptake data by a method described in the text.

TABLE II

THE LOCATION OF C^{14} IN THE ALCOHOL PRODUCED AEROBICALLY BY CORN ROOT TIPS * RESPIRING LABELED GLUCOSE IN THE PRESENCE OF M/200 ARSENITE

	SUBSTRATE		
	GLUCOSE-1-C^{14}	GLUCOSE-2-C^{14}	GLUCOSE-3,4-C^{14}
Amount of glucose added (μM)	100	100	60
Amount of alcohol produced (mg)	2.9	2.4	3.4
Specific activity of glucose **	7.5	25.0	6.2
Specific activity of C in CH_3 of alcohol	1.08	0.15	0
Specific activity of C in CH_2OH of alcohol	0.04	5.03	0
Total radioactivity in CH_3 (mμ c)	1.61	0.19	0
Total radioactivity in CH_2OH (mμ c)	0.06	6.40	0
% of total in CH_3	97.5	2.8	0
% of total in CH_2OH	2.5	97.2	0

* In each case approximately 2-gm root tips were aerated with CO_2-free air in a solution containing the glucose sample and arsenite at pH 5.0. 22.1 mg carrier alcohol were added to each alcohol sample before distillation. The figures are corrected for carrier.

** Specific activity expressed as millimicrocuries per milligram of carbon.

tions. It will be seen that the fates of the constituent C atoms are very close to those which would have been predicted on the E.M.P. scheme. If triose which had arisen from the oxidative pathway had been diverted towards pyruvic acid and alcohol, a different pattern of labeling would have been expected (11); and the relative importance of such a pathway would presumably have determined the masking of the distribution expected on the E.M.P. scheme. The conclusion seems justified that in air, also, glucose dissimilation in corn roots is via the classical glycolytic sequence of reactions.

SUMMARY

From experiments on the fermentation of glucose-1-C^{14} by corn root tips, which produce alcohol and CO_2, the following has been found.

1. Carbon atom 1 (aldehyde carbon) of glucose gave rise to the methyl carbon of ethanol.

2. Both the carbinol carbon of alcohol and the CO_2 contained only traces of the isotope.

From experiments on the oxidation of glucose-1-C^{14}, glucose-2-C^{14} and glucose-3,4-C^{14} by corn root tips in which aerobic fermentation was induced by arsenite and evidenced by alcohol formation, the following has been found:

1. glucose-1-C^{14} yielded methyl labeled ethanol.
2. glucose-2-C^{14} gave rise to carbinol labeled ethanol.
3. glucose-3,4-C^{14} yielded unlabeled ethanol.

These results are compatible with the conclusion that glucose is dissimilated by corn root tips anaerobically as well as aerobically via the classical glycolytic sequence of reactions (Embden-Meyerhof-Parnas pathway).

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THE DIRECT OXIDATION PATHWAY IN PLANT RESPIRATION[1,2]

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An important development in biochemistry has been the discovery of enzymes which can attack glucose-6-phosphate oxidatively to produce pentose phosphate and CO_2. The diversion of glucose-6-phosphate by such a pathway has been variously called the hexose monophosphate shunt and the oxidative pathway and the names of several eminent research workers are linked with the elucidation of the reactions involved (reviewed by Horecker (9)). Recent work on a great variety of tissues, including higher plants (1, 2, 7, 8) has demonstrated that such systems are widespread. In view of this accumulating evidence, some workers have been led to consider that this metabolic "byway" may have importance in addition to its being a probable route to the pentoses in vivo, and the further elucidation of the reactions subsequent to pentose formation have lent color to this view. In particular, the recognition of the importance of sedoheptulose phosphate and its breakdown products has made possible the formulation of a cyclic sequence of events whose operation might of itself account for the total oxidation of glucose (9) and which would, at the least, produce metabolites which are also intermediates in the classical Embden-Meyerhof-Parnas (E.M.P.) glycolytic sequence. Although the details of the reactions remain to be clarified, the recent findings, to which experiments on higher plant tissues have contributed (1, 2, 7, 8), render the operation of the direct oxidation pathway in normal respiration quite plausible. There is ample evidence, of course, for the operation of the E.M.P. sequence of reactions in plant materials (11); at the present time, however, no evidence concerning the relative importance of the two alternatives in plant respiration has been offered. The present investigation was carried out to obtain evidence bearing on this point.

Use was made of the fact that the method of glucose breakdown will determine the relative rates of release of its constituent carbon atoms. (For a discussion of the application of this principle see Bloom, Stetten, and Stetten (5) and Bloom and Stetten (6)). If the glucose molecule were broken down by glycolysis which includes equilibration at the triose level, two pyruvate molecules would be produced in which carbon atom 1 (C-1) and C-6 of the glucose would appear as the methyl carbons of the acid, C-2 and C-5 as the carbonyl carbons, and C-3 and C-4 as the carboxyl carbons. Carbons 1, 2, and 3 of the original glucose would, therefore, be indistinguishable respectively from 6, 5, and 4, and in the subsequent oxidative breakdown of the pyruvate, each member of a pair would appear in the respiratory CO_2 at the same rate as its partner. (Of the pairs, (C-3 and C-4) would be expected to appear first in the CO_2 followed by (C-2 and C-5) and then (C-1 and C-6).) Thus, if comparable samples of tissue were respiring on glucose-1-C^{14} and glucose-6-C^{14} respectively, the contribution of C^{14} to the CO_2 given off would be the same in each case. If, on the contrary, a glucose molecule was metabolized by way of the oxidative pathway, the CO_2 from the glucose-1-C^{14} would be expected to be initially higher in C^{14} than that from the glucose-6-C^{14} since C-1 of the glucose molecule is the first to be converted to CO_2. Provided that no assimilation of carbon residues containing different amounts of C-1 and C-6 occurred, the total yield of $C^{14}O_2$ from the two glucose samples would be the same when oxidation was complete regardless of the path of breakdown. In the present experiments, it should be stressed, the time intervals were such that only a few percent of the supplied glucose was respired and the yields of $C^{14}O_2$ are, therefore, regarded as initial as distinct from total. In these experiments, then, initial yields of $C^{14}O_2$ from 2 glucose samples were compared, and the ratio, % radiochemical yield from glucose 6-C^{14}/% radiochemical yield from glucose 1-C^{14} evaluated. Clearly, a ratio of near unity would indicate that schemes other than E.M.P. were playing a very minor part in glucose breakdown, whereas a ratio of less than unity would implicate the participation of the direct oxidation pathway.

MATERIALS AND METHODS

PLANT MATERIAL: Root tips from corn were prepared as described earlier (3) and 1 gm fresh weight was used as a sample.

Peas (var. Alaska) were grown on gravel in the greenhouse and the other plant materials (see table), with the exception of carrot, were from stock greenhouse plants. Carrots were purchased locally. The procedure in each case was to cut the experimental material into sections or pieces about 1 mm in thickness. These were washed in several changes of water, dried lightly and weighed out into replicate samples of 1 to 2 gm fresh weight for transfer to Warburg vessels.

MANOMETRIC EXPERIMENTS: In most of these experiments four equal samples of tissue were placed in Warburg vessels of 100 ml capacity. The large vessels permitted the use of a relatively large volume of tissue and a small liquid volume (2.5 to 4.0 ml). The liquid contained 1 ml of 0.03 M phosphate buffer at pH 5.0, plant tissue and water. To two of the four vessels 20 to 40 micromoles of glucose-1-C^{14} of known activity were added while the others received the same amount of glucose-6-C^{14}. The glucose was

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[2] Research carried out at the Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

added immediately prior to attaching the vessels. The vessels were shaken at 25° C for a period of 4 to 5 hours during which the light was excluded in the case of green tissues. The respired CO_2 was absorbed in 0.2 ml 20 % KOH in the center well. The values for CO_2 determination from the four samples, which include errors in sampling, pipetting, transfer and conversion to $BaCO_3$ showed that the technique was adequate; the spread between four such values was usually considerably less than 10 %.

Measurements of Radioactivity: At the end of the experiments the KOH was removed and the absorbed CO_2 was converted to $BaCO_3$ by a method described by Steele and Sfortunato (10). The radioactivity of the $BaCO_3$ was measured with a methane flow proportional counter.

The glucose-1-C^{14} was kindly supplied by Dr. H. Isbell of the National Bureau of Standards. The glucose-6-C^{14} was kindly given to us by Dr. J. C. Sowden, Washington University of St. Louis.

Results and Discussion

From the primary data the radiochemical yield in millimicrocuries from each glucose sample was evaluated and this was then expressed as a percentage of the total radioactivity supplied in the original glucose. These values showed that, under the experimental conditions, only a small fraction of the glucose had been respired (usually less than 10 % of the C-1 carbon having appeared). This permitted the evaluation of initial C_6/C_1 ratios as shown in table I, which includes values obtained from a variety of plant tissues.

It is significant that the value of the C_6/C_1 ratio for excised corn root tips was near unity in each of three experiments, since this is a value which indicates that the oxidative pathway, if it does operate in the normal respiration, plays a very minor role (3). A point of major importance is that in contrast to the corn root tips, most of the tissues tested gave values of the ratio which were considerably less than unity and even in some cases less than 0.5. In these tissues, then, although the actual rates of CO_2 output on the 2 kinds of glucose were the same, the ratio shows that in the early stages of glucose utilization, C-1 was making a significantly greater contribution to the CO_2 than C-6. This is what would be expected if a portion of the glucose followed the oxidative pathway.

Indeed, if we adopt the procedure outlined by Bloom and Stetten (6) and Bloom et al (5) we can arrive, with similar reservations, at a limiting estimate of the amount of glucose which was actually being respired by pathways other than E.M.P. Clearly if glucose catabolism occurs simultaneously by: (a) the E.M.P. pathway, with equal contributions to the CO_2 coming from C_1 and C_6 of the glucose, and (b) the oxidative pathway, in which a rela-

Table I

Evaluation of C_6/C_1 * Ratio for Various Plant Materials

Tissue	Glucose	Mμ C added	Mμ C recovered	% recovered	C_6/C_1 = Max. fraction of E.M.P.
Corn root	1-C^{14}	32.0	1.18	3.7	0.91 (1.12, 0.95) **
	6-C^{14}	33.9	1.13	3.4	
Pea internode	1-C^{14}	16.0	0.40	2.5	0.48
	6-C^{14}	17.0	0.20	1.2	
Pea leaf	1-C^{14}	10.2	0.57	5.5	0.61
	6-C^{14}	8.5	0.29	3.4	
Sunflower leaf	1-C^{14}	13.4	2.42	18.1	0.75 (0.63) **
	6-C^{14}	10.2	1.38	13.5	
Carrot petiole	1-C^{14}	13.4	0.82	6.1	0.36
	6-C^{14}	13.6	0.30	2.2	
Carrot root discs	1-C^{14}	13.4	0.51	3.8	0.50
	6-C^{14}	13.6	0.26	1.9	
Bryophyllum leaf	1-C^{14}	13.4	0.18	1.3	0.54
	6-C^{14}	9.2	0.06	0.7	
Corn leaf	1-C^{14}	13.4	0.78	5.8	0.79
	6-C^{14}	9.2	0.42	4.6	
Pea root (3 week old system)	1-C^{14}	13.4	1.49	11.1	0.58
	6-C^{14}	9.2	0.59	6.4	
Sunflower stem	1-C^{14}	13.4	1.05	7.9	0.75
	6-C^{14}	10.2	0.60	5.9	
Parsley leaf	1-C^{14}	13.4	0.65	4.8	0.65
	6-C^{14}	10.2	0.32	3.1	
Parsley stem	1-C^{14}	13.4	1.00	7.5	0.64
	6-C^{14}	10.2	0.49	4.8	

* % radiochemical yield from glucose-6-C^{14}/% radiochemical yield from glucose-1-C^{14}. Equal amounts (usually 30 micromoles) of glucose were supplied to samples of tissue in Warburg vessels at 25° C, and the CO_2 produced in a 4 hour experiment analyzed as described in the text.

** The values in parentheses are from individual experiments carried out on separate occasions.

tively greater contribution is initially made by C_1, the *maximum* amount of the $C^{14}O_2$ from glucose-1-C^{14} which can have been produced by E.M.P. is an amount equal to the total $C^{14}O_2$ from glucose-6-C^{14}. Thus the *maximum* fraction of the $C^{14}O_2$ from glucose-1-C^{14} which could have arisen from E.M.P. and hence the maximum fraction of the respiration which could be occurring through this pathway is given directly by the C_6/C_1 ratio and the *minimum* contribution of the oxidative pathway can be derived directly from this figure. The figures in the last column of table I therefore show that in most of the species tested the contribution of the oxidative pathway was a very considerable one.

Two points about the values of the C_6/C_1 ratios less than one are pertinent. First, it is not suggested that the values are rigid, since, as it has been pointed out elsewhere (4) they would be expected to approach one as the glucose was depleted and C-6 made an increasingly greater contribution to the CO_2. Second, individual species will no doubt differ in the relative efficiencies with which they accomplish reactions in the oxidative pathway prior to and subsequent to C_1 release. Since it is the difference in the rates of these reactions which determines what the C_6/C_1 ratio will be, it is clearly possible that two plant species in which the fraction of glucose entering the oxidative pathway was the same might, nevertheless, yield different ratios and hence different values for the *maximum* contribution of the E.M.P. pathway.

The list of tissues tested is certainly too small to warrant detailed deductions, and the values would have been more reasonably compared if they all could have been obtained at the same stage in the disappearance of added glucose. Nevertheless, it seems reasonable to conclude that the oxidative pathway plays a considerable role in plant respiration and that in none of the tissues tested except young corn root tips was the Embden-Meyerhof-Parnas scheme the sole means of glucose breakdown in respiration.

Summary

Experiments have been carried out on a variety of plant tissues to find whether the oxidative pathway of glucose breakdown ("hexosemonophosphate shunt") contributes to their respiration. When initial yields of $C^{14}O_2$ from equal amounts of glucose-1-C^{14} and glucose-6-C^{14} were compared there was clear evidence that, in most of the tissues, some of the glucose was, in fact, broken down in reactions in which C-1 was split off at an earlier stage than C-6. It has been calculated from the data that in several of the tissues a maximum of about half of the glucose was broken down by the E.M.P. pathway. The fact that the initial yields of $C^{14}O_2$ from the two kinds of glucose were the same when corn root tips were used confirms an earlier finding that in this juvenile tissue the classical glycolytic route is the sole one; in the other tissues it is concluded that a substantial fraction of the glucose is respired via the direct oxidation pathway.

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INVESTIGATIONS INTO THE FUNCTION OF PYRIDOXIN AS A GROWTH FACTOR FOR EXCISED TOMATO ROOTS[1]

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Thiamin, pyridoxin, niacin, and glycine have all been reported to act as growth factors for excised tomato roots grown in sterile culture. White (9) showed that, in basal medium plus thiamin, partial replacement of pyridoxin by glycine could be effected using any one of three different clones of excised roots. The degree to which glycine replaced pyridoxin varied with the different clones. Furthermore, it appears from photographs of the roots that basal medium plus pyridoxin and glycine gave a greater level of growth than was obtained in basal medium plus pyridoxin only. It was shown by the present author (1) that, after the requirement for thiamin was met, glycine plus niacin replaced pyridoxin using root cultures derived from either one of two inbred lines of tomato and their reciprocal crosses. The addition of glycine to basal medium plus thiamin and pyridoxin exerted an inhibitory effect with one of the parent strains and the hybrids but was, if anything, beneficial to growth of the other parent strain.

In view of the many functions exerted by components of vitamin B_6 in co-enzymes (7) it is unlikely that all processes requiring vitamin B_6 in excised tomato roots are eliminated when glycine (or glycine plus niacin) is used to replace pyridoxin. It is more probable that when this replacement of pyridoxin is effected, growth occurs either 1) because glycine plus niacin exerts a "sparing action" upon the amount of vitamin B_6 synthesized by the roots or 2) because glycine and niacin are involved in the biosynthesis of vitamin B_6. This latter possibility is of particular interest as no suggestions as to the pathway of this biosynthesis have been established (11). Thus a detailed study of the replacement may be expected to yield information upon either the function, or the pathway of biosynthesis, of vitamin B_6 in higher plant material.

Glycine, at least in part, replaced pyridoxin in the nutrition of the clone of excised tomato roots studied here. This paper describes other nutritive requirements and interrelationships of the clone. The object of the work was to obtain detailed information on the requirement of the clone for pyridoxin (or other components of vitamin B_6), niacin or niacinamide and glycine.

MATERIALS AND METHODS

The clone of excised roots used as source of inocula for all experiments described here was derived from a single seed of an inbred line of *Lycopersicon esculentum* Mill. var. Red River. It is designated R5. The inorganic constituents of the basal medium used for maintenance of the clone and in experimental media are as given by White (10). Preliminary experiments showed that R5 could not be subcultured indefinitely in basal medium plus thiamin only. Addition of pyridoxin to basal medium plus thiamin permitted continuous subculture and further addition of niacin increased the level of growth. Sucrose supplied in the medium at a concentration of 1.5 % (2) was found to be more satisfactory than the 2.0 % as used by White (10) and others. The stock cultures of R5, therefore, were maintained in a medium containing the inorganic constituents as given by White (10), sucrose at a concentration of 1.5 % and the growth factors thiamin, pyridoxin, and niacin at the concentrations given by White (10) for his standard medium. All experimental media contained sucrose at a concentration of 1.5 %. Double distilled water, the second distillation being from Pyrex glass, was used for all media. The chemicals were the purest obtainable commercially and all glassware was Pyrex.

The passage lengths for stock cultures were four days for the segment passage and six days for the tip passage. Tips 10 mm in length were excised from 4-day-old segments, inoculated one tip per flask (125 ml Ehrlenmeyer) and incubated at $28 \pm 1.0°$ C in the dark. Each flask contained 50 ml of medium sterilized by autoclaving at 15 lbs. pressure for 15 minutes. All cultures were harvested on the sixth day after inoculation. In any one experiment the initial pH of the medium was adjusted, where necessary, so that the pH of each series was approximately the same and within the range 4.8 to 5.0. The method of estimating growth was as described previously (3) except that an estimate of lateral growth was obtained by recording the total lengths of the ten basal laterals on each root.

For convenience' sake the growth factors thiamin, pyridoxin, niacin, and glycine are referred to as T, P, N, and G respectively. Thus TP-medium is basal medium (inorganic salts plus sucrose) supplemented with thiamin and pyridoxin.

RESULTS

REPLACEMENT OF PYRIDOXIN BY PYRIDOXAL OR PYRIDOXAMINE: The compounds pyridoxin, pyridoxal, or pyridoxamine are all known to manifest vitamin B_6 activity and are interchangeable in the nutrition of certain micro-organisms (11). The experiment described here was designed to determine whether these compounds are interchangeable in the nutrition of R5.

Pyridoxin and pyridoxal were supplied as the hydrochlorides and pyridoxamine as the dihydrochloride. Each compound was tested over a range of concentrations in which the standard concentration (1.0) of the free base was at 4.9×10^{-7} M. Ten replicates were used at each concentration. The results

[1] Received November 10, 1953.

are shown in figure 1, which also includes a detailed range of concentrations of pyridoxin.

At appropriate concentrations the same level of growth resulted with either pyridoxin, pyridoxal, or pyridoxamine. The order of activity, on a molar basis, was pyridoxal > pyridoxin > pyridoxamine. Number of laterals per root was correlated with length of the main axis. With all three compounds the concentration required to give maximum growth in length of the main axis was lower than that required to give maximum growth of laterals.

Replacement of Niacin by Niacinamide: Niacin and niacinamide were tested over a range of concentrations in the basal medium plus thiamin and pyridoxin. The standard concentration was 4×10^{-6} M. Each treatment comprised twenty replicates. The experiment was repeated on a second occasion and gave results which were the same as those shown in figure 2.

Both niacin and niacinamide, at appropriate concentration, resulted in a higher level of growth than occurred in TP-medium. The increase in length of the main axis with niacin at 1.0 times standard was greater than with that obtained in any concentration of niacinamide. This occurred in both experiments. The greatest effect of adding niacin or niacinamide to TP-medium was on the growth of laterals. Growth of laterals with either niacin or niacinamide was approximately double that of the controls. No difference was found in the greatest growth of laterals obtained with niacin or niacinamide. Thus both compounds exerted the same effect on growth of these excised roots. Stimulation of growth of the main axis was slight compared to stimulation of growth of laterals.

The concentration of niacinamide required for maximum stimulation of growth of the main axis was lower than that required when using niacin. The greatest number of laterals per root, or length of laterals per root, was obtained at the same concentration of niacin and niacinamide (1.0 times standard). However, stimulation occurred at lower levels of niacinamide than niacin. Thus niacinamide was, in general, more active than niacin although this point is complicated by the difference between responses shown by the main apex and the lateral apices.

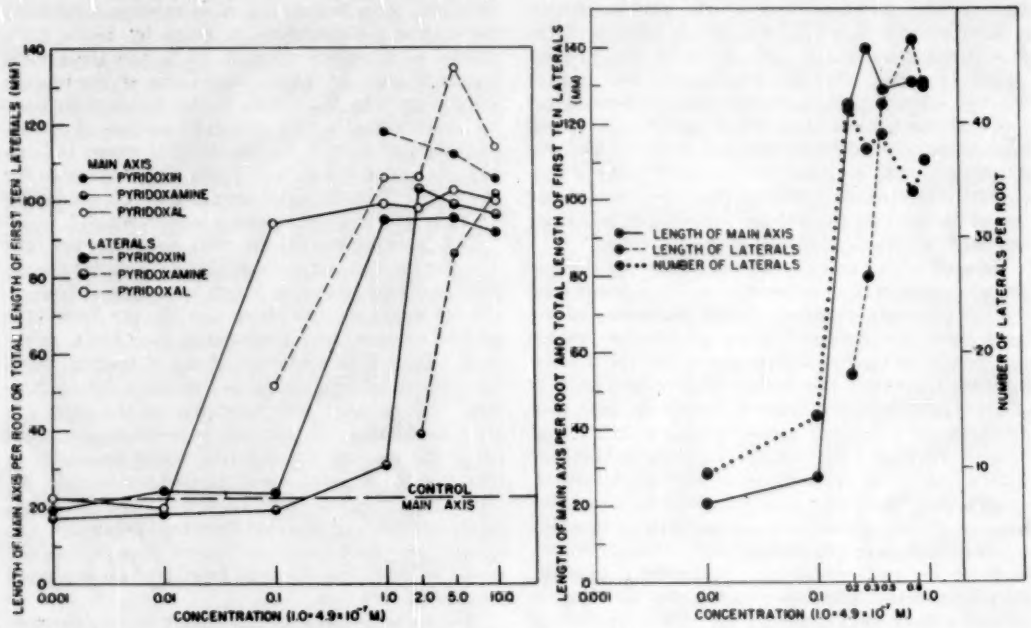

Fig. 1. The effect of the concentration of either pyridoxin, pyridoxal, or pyridoxamine, added to the basal medium plus thiamin (control), on the growth of excised tomato root.

Replacement of Pyridoxin by Glycine or Glycine Plus Niacin: This experiment was designed to test the effect of glycine concentration when added to T-medium and TN-medium. The effect of glycine was tested at four levels of niacin. The experiment was repeated three times. The results were somewhat variable but some effects were consistent from experiment to experiment. The results of one of the experiments are shown in figure 3. Each test comprised fourteen replicates.

In the absence of niacin the optimal concentration of glycine was at 1.0 or 2.0 times standard (standard $= 4 \times 10^{-5}$ M). Toxic effects were manifested at concentrations greater than 2.0 times standard.

The addition of niacin to a medium containing 2.0 times standard glycine or higher did not affect

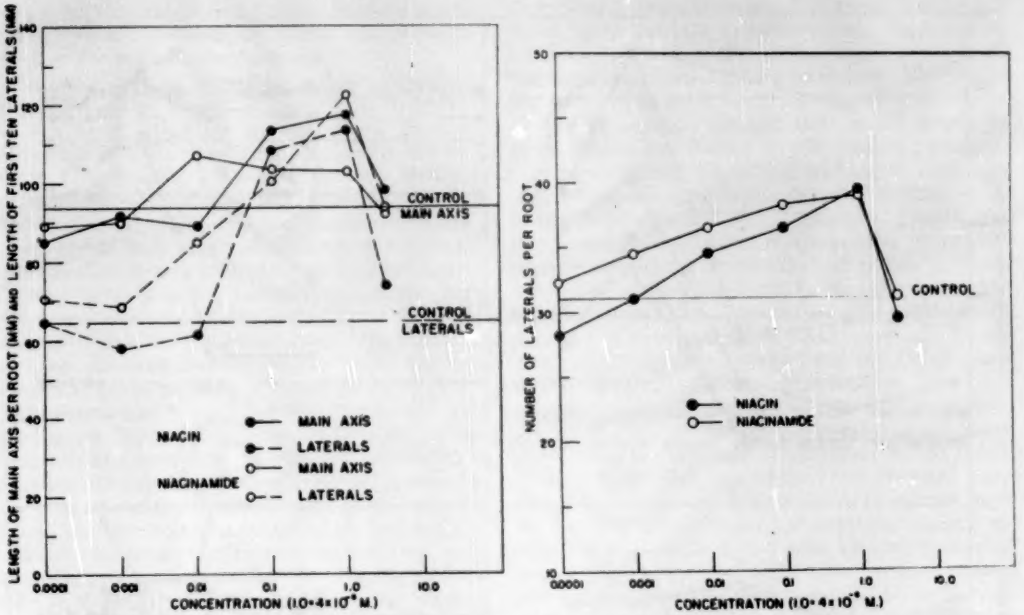

FIG. 2. The effect of the concentration of either niacin or niacinamide, added to the basal medium plus thiamin and pyridoxin (control), on the growth of excised tomato roots.

growth. The increase in the number of laterals per root on making this addition, as shown in figure 3, did not occur in all experiments. At lower concentrations of glycine (0.5 and 1.0 times standard), growth was markedly affected by the addition of niacin. Growth in length of the main axis increased with the concentration of niacin up to the highest concentration tested and the best growth was greater than that obtained in TG-medium. The effect on the growth of the laterals was more variable and differed from that on the growth of the main axis. In two of the experiments growth of the main axis was negligible at 0.5 of standard glycine except on addition of 5.0 times standard niacin. A comparison to show effects of the niacin concentration on lateral growth at 0.5 of standard glycine cannot, therefore, be made. However, at 1.0 times standard glycine there was an increase in the lateral growth with an increase in

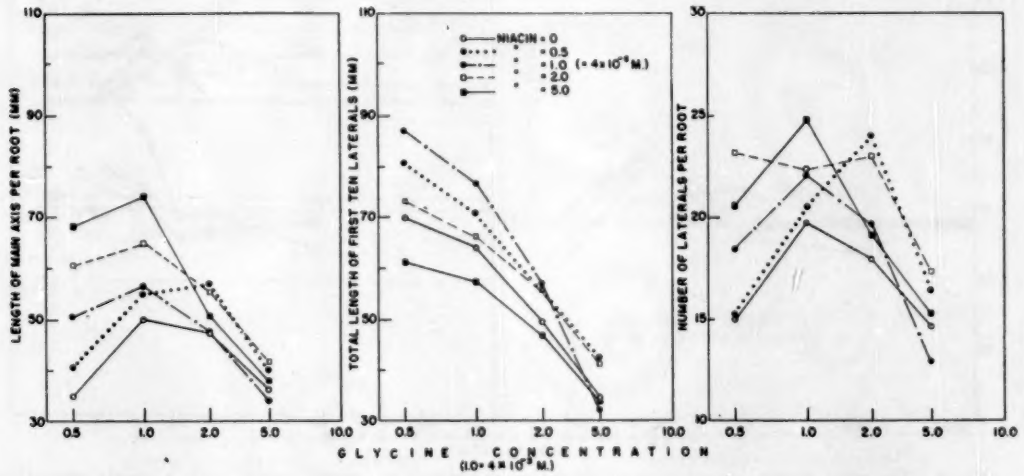

FIG. 3. The effect of the concentration of glycine, in the absence of niacin and at various levels of concentration of niacin, on the growth of excised tomato roots in a medium not containing pyridoxin.

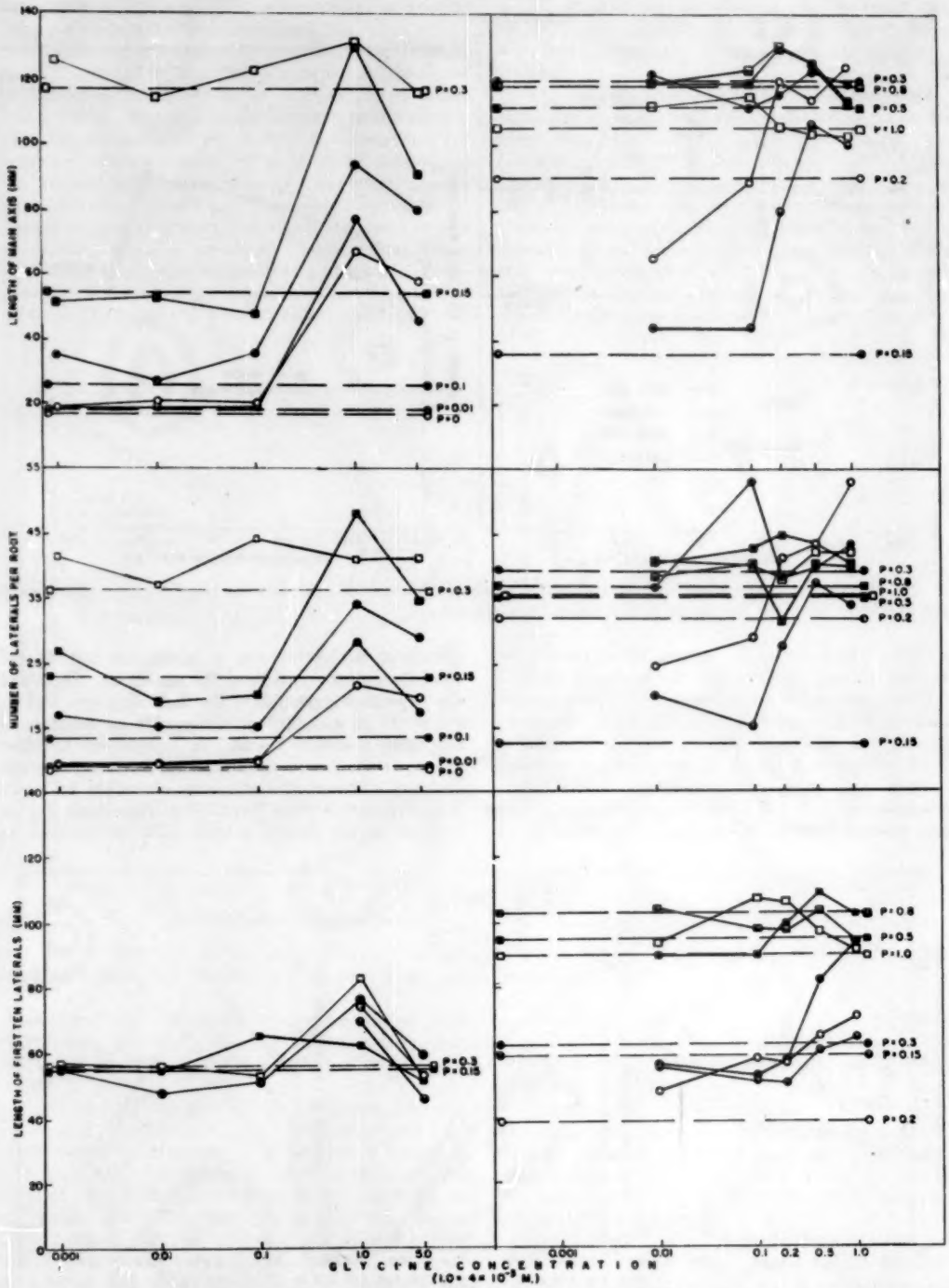

FIG. 4. The effect of the concentration of glycine on the growth of excised tomato roots in media containing various levels of concentration of pyridoxin. The horizontal broken lines show the level of growth in the absence of glycine at the various levels of pyridoxin. (Standard concentration of pyridoxin, $1.0 = 4.9 \times 10^{-4}$ M of the free base.)

niacin concentration up to 1.0 times standard niacin. With a further increase in niacin concentration, growth of the laterals was decreased.

The variability of the results and the differential effects upon lateral and main axis growth mean that the data are of little value for determining the most satisfactory levels at which to supply niacin and glycine in TNG-medium. However, the data do show that a deficiency in glycine could be compensated for by increasing the concentration of niacin. "Optimal" growth of the main axis was obtained at 0.5 or 1.0 times standard glycine with 5.0 times standard niacin. "Optimal" growth of the laterals was obtained with glycine and niacin at 1.0 times standard. It should be noted that in TPN-medium (page 326) niacin at 5.0 times standard reduced growth below that obtained with 0.1 to 1.0 times standard niacin.

INTERRELATIONSHIPS BETWEEN PYRIDOXIN AND GLYCINE IN BASAL MEDIUM PLUS THIAMIN, PYRIDOXIN, NIACIN AND GLYCINE: Under the conditions obtaining when the experiments described in this section were started it was found that growth of clone R5 was not increased by addition of glycine to TPN-medium. However, preliminary experiments indicated that, at low concentrations of pyridoxin, the addition of glycine produced a marked stimulation of growth. Accordingly, more complex experiments were designed to study this relationship.

The effect of the glycine concentration was tested at ten levels of pyridoxin concentration. The levels of pyridoxin concentration were such that the effect of glycine was tested with pyridoxin (a) absent, (b) below stimulatory levels, (c) at various stimulatory levels, and (d) at a level slightly below the point at which it became inhibitory.

Two experiments were required to test the range of pyridoxin concentrations. The range was from 0 to 0.3 times standard in the first, and from 0.15 to 1.0 times standard in the second, experiment. Each treatment comprised fourteen replicates. The results are shown in figure 4.

The experiments showed that, in all media in which growth was limited by the level of pyridoxin, growth was stimulated by the addition of glycine at appropriate concentrations. At lower limiting levels of pyridoxin (P = 0.01, 0.1 of standard) growth was increased by the addition of glycine but it was not possible to detect a concentration of glycine at which the level of growth was equal to that obtained at the "optimal" pyridoxin concentration. At higher limiting levels of pyridoxin (0.15, 0.2 of standard) the addition of glycine gave growth equal to that obtained at the "optimal" pyridoxin concentration.

The number of laterals per root was, in general, correlated with the length of the main axis. There were, however, three marked exceptions to this result. Thus, in a medium containing 0.8 of standard pyridoxin, increasing the concentration of glycine from 0.1 to 0.2 of standard gave, if anything, a slight *increase* in the length of the main axis but a marked *decrease* in the number of laterals. At a lower concentration of pyridoxin (0.2 of standard), an increase in the concentration of glycine from 0.2 to 1.0 times standard gave no significant increase in length of the main axis but a marked increase in the number of laterals. This effect was also observed with a concentration of pyridoxin at 0.3 of standard when the concentration of glycine was increased from 0.01 to 0.1 times standard.

The small decrease in length of the main axis obtained on increasing the concentration of pyridoxin from 0.2 to 1.0 times standard in TPN-medium was

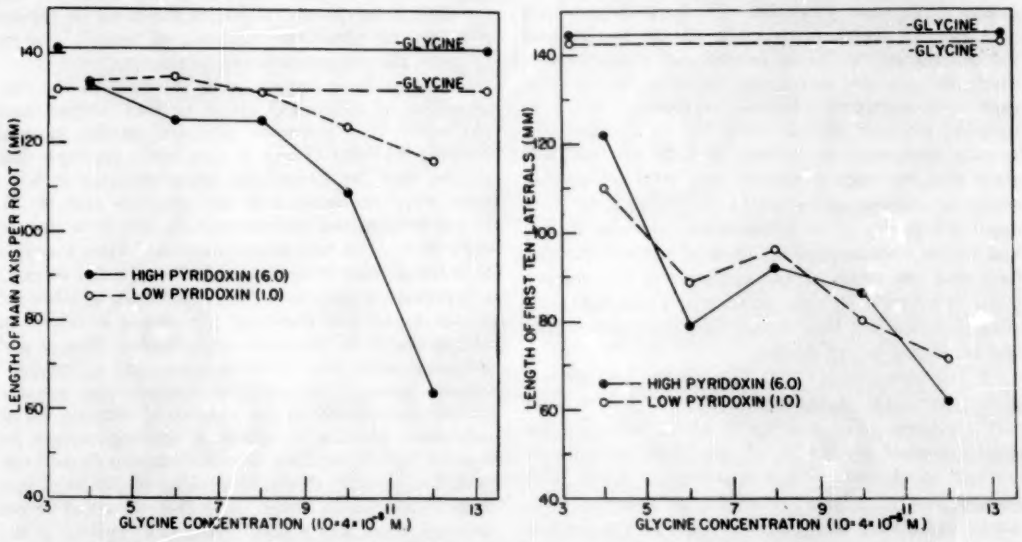

FIG. 5. The effect of high levels of concentration of glycine, at two levels of concentration of pyridoxin, on the growth of excised tomato roots. (Standard concentration of pyridoxin, $1.0 = 4.9 \times 10^{-7}$ M of the free base.)

noted in three separate experiments. A further increase in pyridoxin concentration up to 10.0 times standard showed no significant effect on growth. At low concentrations of pyridoxin (= < 0.3 of standard) glycine was inhibitory at 5.0 times standard concentration. At higher concentrations of pyridoxin (1.0 times standard) glycine was inhibitory at 10.0 times standard concentration. Figure 5 shows the results of an experiment designed to test the effect of a high concentration of glycine at two levels of pyridoxin, 1.0 and 6.0 times standard.

The inhibitory effect of glycine was much more marked on the growth of the laterals than on the main axis. The degree of inhibition of growth of the laterals was the same with either high or low pyridoxin. However, the effect on growth of the main axis showed that inhibition due to a high concentration of glycine in the TPNG-medium was increased by raising the level of pyridoxin to a level which itself was not inhibitory.

Discussion

The clone of excised tomato roots (R5) studied here was found to require thiamin, pyridoxin, and niacin for "optimal" growth although the clone could be maintained in basal medium plus thiamin and pyridoxin. The addition of niacin to this latter medium produced a slight increase in growth of the main axis and a big increase in growth of the lateral roots. Pyridoxin could be replaced by either pyridoxal or pyridoxamine. Niacin could be replaced by niacinamide. The nutritive requirements of micro-organisms for, and the metabolic functions of, these substances are ably reviewed elsewhere (7, 11). The present evidence indicates that B-group vitamins function as components of coenzymes. Pyridoxal and pyridoxamine are the forms of vitamin B_6 which occur in known coenzymes. Organisms which utilize pyridoxin as a growth factor presumably possess mechanisms for converting it to pyridoxal or pyridoxamine. Similarly, nicotinic acid is not known to be a component of a coenzyme whereas nicotinamide is. Presumably nicotinic acid is converted to nicotinamide. If these deductions are correct the data obtained here show that the excised tomato root possesses mechanisms for converting pyridoxin to pyridoxal or pyridoxamine, pyridoxal to pyridoxamine (or vice versa), and niacin to niacinamide. It is of interest to note here that the order of the activities of the components of vitamin B_6 (i.e., pyridoxal, pyridoxin, pyridoxamine) has not been found with any micro-organism or animal so far studied.

It was shown (page 327) that glycine partially replaced pyridoxin in the basal medium plus thiamin and pyridoxin. The addition of niacin increased the effectiveness of glycine at all non-inhibitory concentrations of glycine. Other experiments (page 329) showed that in a basal medium plus thiamin, pyridoxin, and niacin, addition of glycine, at appropriate concentration, stimulated growth at limiting concentrations of pyridoxin to a level equal to that obtained with the "optimal" pyridoxin concentration. It was noted earlier that a limiting level of pyridoxin (or pyridoxal or pyridoxamine) in the basal medium plus thiamin, pyridoxin, and niacin could be used which gave "optimal" growth of the main axis but very little growth of the laterals. At such a limiting level of pyridoxin, the addition of glycine increased growth of the laterals to that obtained with the "optimal" concentration of pyridoxin (see fig 4). An opposite effect was obtained at very low levels of pyridoxin. Thus a comparison of control media (thiamin plus pyridoxin and niacin) containing pyridoxin at concentrations of 0.15 and 0.2 times standard shows that whereas concentration 0.2 times standard gave a greater length of the main axis, the length of the laterals was less than in 0.15 of standard. This effect due to increased pyridoxin concentration was also produced by the addition of glycine to a medium containing 0.15 of standard pyridoxin (see fig 4).

These facts illustrate the similarity of physiological action between glycine and pyridoxin. The close interrelationship was further shown by the fact that the inhibitory effect of glycine at high concentration, in basal medium plus thiamin, pyridoxin, niacin, and glycine, was increased by raising the concentration of pyridoxin to a level which itself was not inhibitory.

Differential effects on the growth of the main axis and the initiation of the laterals (see page 329) were observed on varying the concentration of glycine at limiting levels of pyridoxin. Such differential effects were not observed over the range of concentrations of pyridoxin in basal medium plus thiamin, pyridoxin, and niacin. These facts suggest that glycine exerts an effect on the initiation of laterals. This effect is independent of the relationship between glycine and pyridoxin which affects growth of the main axis and the lateral meristems, although the level of glycine necessary to affect the initiation of laterals was related to the concentration of pyridoxin.

Vitamin B_6 is known to be involved in the condensation of indole and serine to form tryptophane (8) which is a precursor of niacin in the rat and Neurospora (4). There is also some evidence that glycine may be formed by transamination between some unknown amino acid and glyoxylic acid (6, 5). It can be expected that vitamin B_6 will form part of the coenzyme in this transamination. Thus a supply of either glycine or niacin might be expected to exert a "sparing" action upon the vitamin B_6 synthesized by the root. The degree of stimulation obtained on adding niacin to the basal medium plus thiamin and glycine is such that, if niacin is exerting a "sparing" action, niacin would not be expected to increase growth appreciably in the absence of vitamin B_6 or substances exerting a sufficient "sparing" action on vitamin B_6. It has been shown elsewhere (1, and unpublished results) that niacin added to the basal medium plus thiamin failed to produce "normal" roots although there was a slight increase in growth of the laterals and some roots could be subcultured. It

should be noted that the stimulation of growth, particularly of lateral growth, on the addition of niacin to the thiamin-pyridoxin medium indicates that niacin may exert an effect on growth which is not due to any "sparing" action on vitamin B_6.

It is evident that the data obtained so far on the replacement of pyridoxin by glycine, on the synergistic effect of glycine plus niacin in basal medium plus glycine and niacin, and the synergistic effect of glycine plus pyridoxin in basal medium plus thiamin, pyridoxin, niacin, and glycine, demonstrate a connection between vitamin B_6 and glycine or niacin in the clone of excised tomato roots studied here. The metabolic basis of this connection is being examined by analytical and further nutritional studies.

Summary

The clone of excised tomato roots studied here requires thiamin, pyridoxin, and niacin for "optimal" growth but can be maintained in a medium supplemented only with thiamin and pyridoxin. Pyridoxin was replaceable by pyridoxal or pyridoxamine. The order of activity was pyridoxal > pyridoxin > pyridoxamine. Niacin was replaceable by niacinamide. Niacinamide was, in general, the more active.

Pyridoxin could be replaced in part by glycine. This replacement was greater in the presence than in the absence of niacin. In basal medium plus thiamin, pyridoxin, and niacin, the addition of glycine at certain concentrations of pyridoxin increased level of growth to that obtained with "optimal" concentration of pyridoxin.

Morphogenetic effects of glycine and pyridoxin were similar although glycine appeared to exert an independent effect upon the initiation of laterals. Root morphology was controlled by the balance of growth factors supplied in the medium.

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STUDIES ON THE CHEMISTRY OF THE LIVING BARK OF THE BLACK LOCUST IN RELATION TO ITS FROST HARDINESS. VII. A POSSIBLE DIRECT EFFECT OF STARCH ON THE SUSCEPTIBILITY OF PLANTS TO FREEZING INJURY[1,2]

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Under conditions of low autumnal temperatures not far removed from the freezing point, an increase in the frost hardiness of plants is often observed which is accompanied by a disappearance of the starch and an increase in the soluble sugar contents of the perennial tissues. This response is well exemplified in the seasonal changes in the living bark tissue of the black locust tree during late fall (8). It is not surprising that much of the extensive literature on frost hardiness in plants is devoted to a consideration of the extent to which this apparent conversion of starch to sugars may be involved in the actual frost-hardening mechanism. Of the many theories advanced in this regard, nearly all have ascribed the effect to the increase in concentration of the sugars which are produced. Theories of frost hardiness based wholly or in part on the effect of in-

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creased sugar concentration seem attractive from the physico-chemical point of view because of the relation of sugar concentration to a lowering of the freezing point of the cell contents or to a possible stabilizing or protective effect on cell colloids. These theories and the evidence in support of them have been discussed in recent reviews on frost hardiness (3, 4). As a first step in the appraisal of the possible role of soluble sugars in hardiness, it would appear that the degree of correlation between the soluble sugar concentration in the cell and the hardiness of the tissue would have been thoroughly examined. If sugar concentration were an important factor in frost hardiness, a positive correlation between these factors would be maintained and observed at all times and under all conditions. Yet when this relationship was examined in the cells of the bark of the black locust under seasonal as well as unseasonal conditions (5) such a positive correlation was not observed. A similar lack of correlation between the hardiness and the sugar content or the osmotic pressure of the tissues of other plants is reported generally in the literature (1, 2, 3).

Regardless of whether sugar concentration is a factor in the frost hardening mechanism, a possible direct effect of the elimination of starch from the cells on their frost hardiness has been either overlooked or discounted. Indeed Levitt (3), after examining the controversial evidence pertaining to the relation of soluble sugars to hardiness and the effect of starch to sugar transformations on hardiness, poses the question, "might not the presence of starch be harmful rather than the presence of sugars beneficial?" The possible injurious effects of starch contained in cells of tissues when they are frozen and, conversely, the beneficial effects which would be derived, if such an injurious effect exists, from the prior transformation of this starch to sugar, should have been suspected in view of the recent evidence of the mechanical nature of frost injury (4).

A deleterious effect of starch on cell hardiness became increasingly suspect in the course of studies on other aspects of the chemistry of the hardiness mechanism in the black locust when it was noted incidentally that a definite measure of increased hardiness consistently accompanied any considerable decrease in the starch content of the bark cells of this tree as determined by iodine staining of tissue slices. An investigation was therefore undertaken to examine more closely the relationship between the variations in the carbohydrates of the black locust and its frost hardiness, with special reference to the role of starch. A part of this study, describing analytically determined variations in the starch and soluble sugar contents of locust bark tissue under a variety of seasonal and unseasonal conditions in normal and ringed trees and in cut logs, has been presented earlier (8). The present paper is concerned with the relationship of these variations in the carbohydrates to the variations which were observed at the same time in the hardiness of this tissue.

Methods and Results

The analytical procedures employed in the estimation of sugars, starch, and water soluble protein contents of the bark tissues are those described earlier (7, 8). Frost hardiness of the tissues was measured by the plasmolysis test (6) and is reported either in terms of the highest molarity of salt solutions ($NaCl : CaCl_2 :: 9 : 1$) to which the tissue could be subjected before experiencing a 50% killing of the cells, or in terms of the percentage survival of the cells when the tissues were subjected to salt solutions of varying molarity.

Variations in the hardiness of locust bark tissue have been shown to be associated principally with changes in the water soluble protein content of the

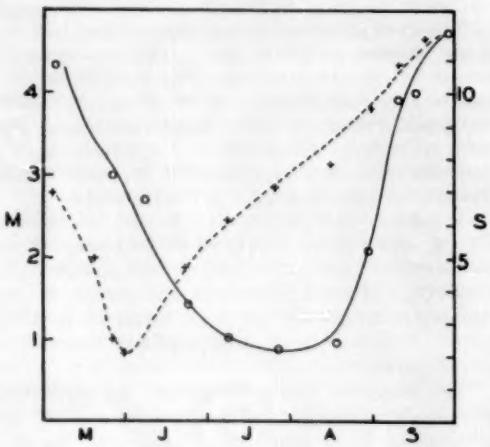

Fig. 1. Total sugar content (---+---) and degree of hardiness (---○---) of living bark tissues at various times during summer. Hardiness measured as molarity (M) of salt solutions required to effect 50% killing of cells in plasmolysis. Sugar content (S) measured as % of dry weight of tissue. Abscissa indicates dates (May to September, inclusive) at which samples were taken.

cells (7). In order to detect any limiting relationship which might exist between hardiness and the starch or sugar content of the cells it is necessary, therefore, that concomitant variations in their water soluble protein content be taken into account.

Soluble Sugar Content versus Hardiness of Bark Cells: Several lines of evidence have been obtained which indicate that no primary relationship exists between the soluble sugar content of a locust bark cell and its hardiness. For example, when the courses of the normal variations in the total soluble sugars and in the frost hardiness of the bark of intact trees are followed simultaneously throughout the summer season, results such as those illustrated in figure 1 are obtained. It is evident that the amount of soluble sugars existing in the bark tissues at any time bears little, if any, relationship to its frost

hardiness at that time. In late spring the hardiness which existed previously in winter is still decreasing while the sugars have already passed a minimum and begun to accumulate. The sugars continue to increase steadily and progressively throughout June and July while hardiness passes through the minimum for the year. Fairly high levels of soluble sugars are reached in July, long before the hardiness for the following winter has begun to develop. It has already been shown (5, 7) that the trends exhibited here in hardiness follow closely the trends in the concentration of water soluble protein. In early spring, hardiness is slow to disappear in the bark. It does so in proportion to the extent to which the winter accumulation of water soluble protein disappears. Yet appreciable depletion of soluble sugars occurs very early in the spring by virtue of the apparent conversion of these sugars to starch (8) and by respiration. For these reasons the level of soluble sugars in the tissues in early May is much the same as in late July although the hardiness of the bark in May is much greater. A comparison of May and July bark tissues in these respects as well as in respect to the water soluble proteins and starch is shown in table I. But for the considerable accumulation of starch in the May tissues it is probable that their hardiness differences would be even greater.

TABLE I

THE RELATION OF SOLUBLE SUGAR, STARCH, AND WATER SOLUBLE PROTEIN CONCENTRATION TO FROST HARDINESS IN THE BARK OF NORMAL TREES IN JULY AND MAY

DATE	% DRY WEIGHT			MOLARITY OF PLASMOLYZING SOLUTION REQUIRED FOR 50 % INJURY
	SOLUBLE SUGAR	STARCH	WATER SOLUBLE PROTEIN N	
July 25	6.8	1.2	0.58	1
May 15	6.3	5.8	1.00	4

If a tree is doubly ringed down to the wood in mid-summer so as to intercept, from above and below, a section of the bark on the trunk, translocation of carbohydrate to or from this section is arrested. It was shown (7) that if this ringing is performed in mid-summer and the variations in hardiness and soluble sugars are followed simultaneously in these sections from the time of ringing until early winter, the level of soluble sugar drops while that of the hardiness increases, although a degree of hardiness equal to that of normal trees is never reached. The hardiness tests and carbohydrate analyses made on such sections consistently indicate that the change in hardiness and the change in soluble sugars occur in opposite directions. Table II shows these variations in two such sections.

Conversely, if a tree is doubly ringed in winter and the tissues of the section of bark so intercepted are tested for hardiness and analyzed in June, it is found that while the soluble sugars in this section have been depleted to almost the same extent as in normal trees at this time, the loss in hardiness is much less than in the bark of normal trees. This is illustrated in table III. The unusual retention of hardiness in June in such winter ringed sections occurs independently of the loss of soluble sugars and is associated with the retention of a level of water soluble protein more characteristic of winter tissues (5). Comparison of the level of sugars existing in the hardy ringed tissue of June 23 in table III with that in the normal unhardy tissues of July 25 in table I further emphasizes the lack of correlation of soluble sugar content with hardiness. These observations indicate that it is unlikely that soluble sugar concentration per se is a primary factor in frost re-

TABLE II

CHANGES OCCURRING IN THE FALL IN THE SOLUBLE SUGARS, STARCH, WATER SOLUBLE PROTEIN, AND FROST HARDINESS IN THE BARK OF TRUNK SECTIONS OF TWO TREES INTERCEPTED BY DOUBLE RINGING IN MID-SUMMER

TREE NO.	DATE OF SAMPLING OF SECTION	% DRY WEIGHT			MOLARITY OF PLASMOLYZING SOLUTION REQUIRED FOR 50 % INJURY
		SOLUBLE SUGAR	STARCH	WATER SOLUBLE PROTEIN N	
3-R	*Initial* section ringed and sampled July 25	8.3	1.1	0.66	2.5
3-R	*Final* Sampled Nov. 10	5.3	0.3	1.05	4.5
4-R	*Initial* section ringed and sampled Aug. 15	7.3	1.8	0.55	0.5
4-R	*Final* Sampled Nov. 15	4.6	0.3	0.65	2.7

TABLE III

THE RELATION OF SOLUBLE SUGAR, STARCH, AND WATER SOLUBLE PROTEIN CONCENTRATION TO FROST HARDINESS ON JUNE 23 IN THE BARK OF A NORMAL TREE AND IN THE BARK OF A SECTION OF TRUNK ISOLATED BY RINGING IN WINTER

DATE	DESCRIPTION	% DRY WEIGHT			MOLARITY OF PLASMOLYZING SOLUTION REQUIRED FOR 50 % INJURY
		SOLUBLE SUGARS	STARCH	WATER SOLUBLE PROTEIN N	
Feb. 6	Normal winter bark	12.8	0.1	1.29	>5
June 23	Section of trunk isolated by ringing in Feb.	6.4	0.5	1.25	5
June 23	Normal tree	4.0	0.9	0.57	1.5

FIG. 2 a and b. Photomicrographs of iodine-stained normal locust bark tissue of (a) (*left*) high and (b) (*right*) low starch content.

sistance. Is, then, the association of a measure of increase in hardiness which is observed to accompany the replacement of starch by soluble sugars in the tissue purely fortuitous, with both processes occurring independently but at the same time seasonally? The following considerations and evidence would indicate that this is not entirely so.

THE EFFECTS OF STARCH ON HARDINESS: Starch is observed to occur in considerable quantities in the bark at certain times of the year (8). If, at these times, tissue sections are cut from the bark and stained with iodine, the characteristic and intense blue color is given by the large granules of starch present in the cells. This is illustrated in figure 2a. The intense staining reaction of such tissues is in sharp contrast to that given by those tissues which are completely devoid of starch (fig 2b). When starch is present in such high concentration the granules can occupy a considerable proportion of the protoplast. When such cells of high starch content are placed in strong salt solutions the cells plasmolyze with osmotic reduction in the solvent space of the protoplast but with relatively little reduction in the volume of the insoluble starch granules. The result is that, with contraction of the protoplast, the starch granules within it are packed tightly together and bulge out at its periphery as shown in figure 3a. This too is in contrast to the characteristic smooth indentation or concave plasmolysis of the protoplasts of cells devoid of starch when these are plasmolyzed in the same solutions (fig 3b). Moreover if the cells of both tissues were given time to round up in

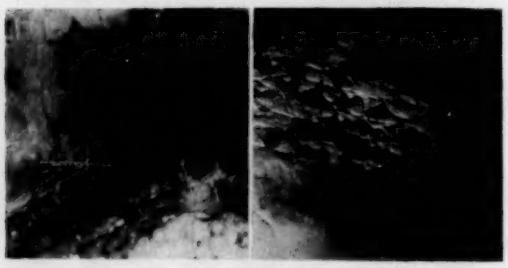

FIG. 3 a and b. Photomicrographs of plasmolyzed locust bark tissue (without staining) of (a) (*left*) high and (b) (*right*) low starch content.

smooth, convex plasmolysis the volume occupied by the protoplasts of cells with high starch would, because of the relative lack of osmotic reduction in the granule size, be greater than that occupied by the protoplasts of cells without starch. The dehydrated and contracted protoplasm and protoplasmic membrane in the starch-containing cells are not only distorted but stretched irregularly by extension over a greater area than in the starch free cell.

Injurious effects produced on bark tissues by the processes of freezing, desiccation, plasmolysis are essentially similar in that the injury in all cases derives from the mechanical stresses exerted on the protoplasm or the protoplasmic membrane as these are rendered highly and even irreversibly deformable by virtue of the dehydrations (6). The effect of these stresses on a stiffened or coagulated protoplasm or protoplasmic membrane can be sufficient to cause rupture, with killing of the cell. As a protective mechanism against these effects of dehydration, the physical properties of the protoplasm of the cells of plants which are developing hardiness are so altered as to render the protoplasm less subject to injury owing either to excessive stresses or to irreversible changes in deformability when dehydrated. The extent to which these favorable properties are acquired in the development of hardiness in the cells of a plant becomes, in effect, a measure of what can be called the intrinsic or real frost hardiness of the plant, and provides the basis for the tests of frost resistance which have been described (6). At any stage of intrinsic frost hardiness of the protoplasm, it is conceivable that the presence of a considerable amount of insoluble starch in frozen, desiccated, or plasmolyzed cells could exaggerate the mechanical stresses incurred and enhance the probability of injury. The following evidence tends to support this hypothesis.

The maximum degree of hardiness which can be attained in those tissues which are completely devoid of starch is never observed in tissues where a considerable accumulation of starch is present, even though the soluble sugar or protein concentrations may be the same in both samples of tissue. Incontroversial evidence for this is, however, difficult to obtain because of the conflicting effects of variations which usually occur simultaneously in the water soluble proteins and in the starch. A striking illustration of this effect should have been obtained from the multiply ringed tree experiments described in a previous paper (7), where, because of arrested translocation, high accumulation of starch was observed to occur in late summer in the bark of those sections of the trunk situated above the uppermost rings. Levels of starch as high as 15 to 20 % of the dry weight of the tissue were encountered in September in these sections, in contrast to the 5 to 7 % level observed in normal trees at this time. A comparison of the hardiness of the bark of these sections with that of the bark of normal trees should have revealed considerable differences in hardiness if starch was effective in promoting injury. The hardiness of these

high starch tissues was indeed found to be lower than that of tissues of normal trees, as is indicated in table IV. Unfortunately the effect of ringing was also to retard the rate of synthesis or accumulation of protein in these ringed sections, as compared with normal trees. Consequently it was not possible definitely to separate, through comparison with normal trees, the effect of high starch from that due to lack of a normal synthesis of protein in these sections.

Alternatively, since the starch levels of late summer become greatly reduced with the incidence of lower temperatures in late fall, it would have been advantageous to follow, in these sections of abnormally high starch content, the corresponding effect on hardiness. However, here again (table IV) the synthesis of protein which occurred in these sections in late fall accompanying the lowering of the starch

TABLE IV

LEVELS, ON SEPTEMBER 21, OF SOLUBLE SUGAR, STARCH, WATER SOLUBLE PROTEIN, AND FROST HARDINESS IN THE BARK OF A NORMAL TREE, IN THE BARK OF A SECTION OF THE TRUNK ABOVE RINGS IN A MULTIPLY RINGED TREE, AND IN THIS LATTER SECTION IN NOVEMBER AFTER FURTHER ISOLATION BY RINGING FROM ABOVE ON SEPTEMBER 21

DATE OF SAMPLING	DESCRIPTION	% DRY WEIGHT			MOLARITY OF PLASMOLYZING SOLUTION REQUIRED FOR 50 % INJURY
		SOLUBLE SUGARS	STARCH	WATER SOLUBLE PROTEIN N	
Sept. 21	Normal tree	9.1	6.8	0.97	4.5
Sept. 21	Section of a ringed tree above rings	7.0	15.6	0.74	2.5
Nov. 10	Same section as above, after having been isolated by ringing from above on Sept. 21	10.2	3.4	1.07	>5

content obscured the effects due to starch which might otherwise have been revealed.

In one group of normal trees, conditions existed in autumn which made possible the direct observation of the effect of starch disappearance at lower fall temperatures upon hardiness. In this group the protein accumulation reached its maximum early in September, before temperatures became low enough to stimulate starch disappearance in the tissues. Thus, while the effect of starch upon hardiness could not be shown to best advantage, because the starch variations are not as considerable in normal as in ringed trees, the effect was rendered more discernible in the absence of any significant change in protein. The results of a comparison in tissues from one such tree are shown in table V. Neither the differences in the soluble sugar level nor in the protein level will serve adequately to explain the hardiness changes indicated between these samples. These differences in

TABLE V

CHANGES IN SOLUBLE SUGARS, STARCH, WATER SOLUBLE PROTEIN, AND HARDINESS OCCURRING FROM SEPTEMBER TO MARCH IN THE BARK OF THE SAME TREE

DATE OF SAMPLING	% DRY WEIGHT			% SURVIVAL AT VARIOUS MOLARITIES OF PLASMOLYZING SOLUTION		
	SOLUBLE SUGARS	STARCH	WATER SOLUBLE PROTEIN N	3 M	4 M	5 M
Sept. 29	11.5	6.0	1.06	100	60	10
March 3	10.0	0.1	1.11	100	100	100

hardiness seem accountable only in terms of the variation shown in the starch level and are probably greater than is actually indicated, because 100 % survival at the highest concentration of plasmolyzing solution used (5 M) does not always include the full potential of resistance of a tissue to freezing (6).

Other evidences of an effect of starch on hardiness are adduced from observations made in the spring of the year. An increase in starch content occurs in the bark in spring at the expense of soluble sugars and in response to increases in the average daily temperature (8). During this time (March to June), fluctuations in hardiness were observed to coincide with fluctuations in starch content even though there was little variation in soluble protein—the hardiness decreasing with increasing starch concentration. With persisting higher temperatures at the end of May, there occurred a pronounced increase in the amount of starch, a corresponding drop in the sugar level, and a definite decrease in the hardiness (table VI). This decrease in hardiness could be only attributed either to the increase in starch or to the decrease in the sugar level because of the small variation in protein. This normal process of apparent conversion of sugars to starch, at this time of year, and the associated process of de-

TABLE VI

CHANGES IN SOLUBLE SUGARS, STARCH, WATER SOLUBLE PROTEIN, AND HARDINESS IN THE BARK OF NORMAL TREES IN SPRING AND IN THE BARK OF LOG SEVERED FROM A TREE IN MAY AND STORED FOR ONE MONTH AT +3° C

DATE OF SAMPLING	DESCRIPTION	% DRY WEIGHT			MOLARITY OF PLASMOLYZING SOLUTION REQUIRED FOR 50 % INJURY
		SOLUBLE SUGARS	STARCH	WATER SOLUBLE PROTEIN N	
March 3	Normal	10.1	0.1	1.13	>5
March 30	Normal	11.5	0.3	1.20	>5
May 22	Normal	3.6	7.0	1.12	3.5
June 21	Log severed on May 22 and stored at +3° C	9.8	0.2	1.09	5.0

creasing hardiness could be reversed by holding the tissues at a temperature a few degrees above the freezing point. Thus, when, at the point of maximal accumulation of starch (May 22), a length of trunk with bark intact was severed from the tree and transferred to a refrigerator at +3° C, the starch almost quantitatively reverted to soluble sugars (largely sucrose) and most of the hardiness existing originally in the tree in late winter was restored. On the basis of the evidence which has been presented above, the variations in soluble sugars could not account for the changes noted in the hardiness of the tissues under these conditions. Since variations in protein are not involved, it would appear that the important factor concerned in the early normal drop in hardiness in spring—and in the restoration of this hardiness in the severed log through application of low temperature—is the variation in the starch content of the cells.

Discussion

The capacity of a plant cell to withstand rupture or disruption of internal organization from changes in its protoplasmic volume, due to dehydration and rehydration, is a measure of its winter hardiness against extracellular freezing (6). The hypothesis is proposed that the presence of starch in a cell increases its susceptibility to such injury, the incompressibility of the granules causing the protoplasm to be subjected to greater shearing forces as it is stretched around the granules during dehydration. According to this hypothesis, with other conditions being equal, the presence of starch in a cell will produce an increment of decrease in the winter hardiness. Similarly, a disappearance of the starch will produce an increment of increase in hardiness. It is suggested therefore that the observed increase in hardiness which accompanies the disappearance of starch from the bark cells of the locust tree in autumn, when temperatures approach the freezing point, can be ascribed in part to this circumstance rather than to any effect due to an increase in soluble sugars which usually accompanies this disappearance of starch. The soluble sugar content of the cells has been demonstrated, under a variety of conditions, to manifest no detectable effect upon the winter hardiness of the cells.

While the hardiness increment associated with the presence or absence of starch in the cell would be of a lower order of magnitude than the increment of hardiness associated with the water soluble protein content of the cell (5, 7) it could, under proper conditions of winter temperature variations, mean the difference between death and survival of the tissues. Periods of warm weather in spring are quickly reflected in a resynthesis of starch in the cells (8). This may occur also during warm periods earlier in winter. Should such warm spells be followed by quick and radical temperature drops, cells that would normally be hardy to such temperatures could be killed because of the starch accumulated in the cell during the preceding warm spell. A temperature induced variation in hardiness of trees and shrubs is often observed in the course of hardiness testing in horticultural practice. This may well be related to the reversible temperature-sensitive conversions of starch and soluble sugars and to the postulated direct effects of starch upon the susceptibility of the cells to frost injury. The ease, rate, and degree of reversibility of the conversion process will, of course, vary with the particular species or variety of plant and with the time of year. It is suggested further that differences in the susceptibility of different parts of the same plant may be attributable to localized variations in starch content.

It is obvious that the hypothesis presented in this paper has a bearing on the problem of hardiness only in those plants, like the black locust, where starch constitutes a major reserve carbohydrate.

Summary

With the onset of low autumnal temperatures there is observed a simultaneous decrease in starch content and an increase in soluble sugar content of the living bark cells of the black locust. An increase in soluble protein content precedes the decrease in starch content or continues at the same time. An accompanying increase in frost hardiness of the cells is largely associated with the protein change but under circumstances where the protein change is minimal or zero during the time interval of the carbohydrate conversion some increase in hardiness is observable during the starch-sugar content change.

Evidence is presented that variations in soluble sugars in the bark tissue at other times bear no relationship to the frost hardiness of the cells. It is proposed therefore that the small but discernible hardiness change which accompanies the disappearance of starch is due to a detrimental effect which is exerted by the starch itself, at higher concentrations, on the dehydrated cells. Such a detrimental effect of starch is shown to be possible in view of the mechanism by which frost injury is produced in the cells; the starch granules serve to enhance stress and distortion effects in the protoplasm as engendered, through dehydration, by the freezing process.

It is suggested that readily reversible changes in frost hardiness which have been reported to occur in tissues of some woody plants in response to temperature fluctuations could be due in part to the temperature-sensitive variations in the level of the starch content of these tissues.

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THE AVAILABILITY OF MAGNESIUM FROM ORGANIC CARRIERS IN ARTIFICIAL SUBSTRATES[1,2]

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A mineral deficiency of apple trees known as "leaf scorch" and by various other names occurs very widely in apple growing areas. While the symptoms vary under different environmental conditions intervenal chlorosis of the leaves is most common. These effects develop to scorch, often attaining brilliant tints and may lead to severe defoliation. Mulder (7) illustrates the marginal chlorosis and scorching of foliage of apples grown in Holland. In many New Hampshire orchards, instead of chlorosis, bronzing or brown spotting occurs with no preceding chlorosis. The yield is lowered markedly in some cases, and preharvest dropping is increased.

The literature on the cause and control of leaf scorch has been reviewed recently quite thoroughly by Boynton (3). Most investigators are agreed that it is due to either a deficiency of magnesium in the soil or unavailability of the element. The effects of this deficiency may be aggravated by high amounts of potassium. The various phases of the interdependence of these factors are not clear. Mulder (7) found that the potassium content of leaves from magnesium deficient trees may approach twice the amount present in leaves from a healthy orchard. For control, numerous inorganic carriers of magnesium, such as magnesium sulfate, kieserite, and dolomitic limestone, have been tried in applications to the soil. Recovery of the trees is slow, often with little effect even after three years and with heavy applications. Apparently, under some circumstances, the roots of the apple are unable to absorb much magnesium in these forms from the soil. Spraying of apple foliage or injection of solutions of inorganic magnesium into tree trunks may give some temporary alleviation of the trouble.

That this deficiency is not due to fixation of magnesium in the upper layers of the soil and resulting unavailability to the roots, at least for some soil types, was shown by Lott (6) in his work with magnesium deficiency of grapes in North Carolina. He reported that delayed responses in the plants evidently cannot be attributed to slow movement of magnesium into the root zone and that a portion of the magnesium applied to the soil remains in the surface foot for more than a year. A similar deficiency in grapes was reported by Scott and Scott (8) in Maryland. They also found good penetration of magnesium into the soil from applications of magnesium sulfate, but slight response in correcting the trouble. Spray applications to the foliage gave prompt alleviation. A later paper (9) reported fairly good results from the soil applications but slower recovery than from sprays. Davis and McCall (4) describe a magnesium deficiency in celery, which also does not respond to soil treatment, but is alleviated by spray applications.

It is apparent from this discussion that the usual inorganic sources of magnesium are frequently inadequate to correct this deficiency. Even though spray applications on foliage may help, such relief is temporary and the extra trouble and expense of application make this an unsatisfactory method. The use of organic compounds of magnesium, particularly those in which the metal does not readily ionize from the rest of the molecule, seemed to offer some possibilities in aiding utilization of this element by plants, and ultimately the alleviation of such deficiencies as

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leaf scorch. So far as the authors are aware, only one other instance (6) has been reported on use of organic compounds of magnesium for such purposes.

Preliminary Cultures with Different pH Levels

Materials and Methods: Several different organic compounds of magnesium were tested in preliminary, water and sand, nutrient cultures of corn and apple seedlings. The aim here was twofold: (a) to gain information about the suitability of certain compounds, i.e., whether toxic or not, and (b) to determine pH relationships. Corn plants were used in some of the preliminary cultures because they grow faster; they are also quite sensitive to magnesium deficiency and the symptoms are fairly definite. The list of magnesium compounds used is as follows: thiocyanate, salicylate, lignin sulfonate, glycerophosphate, ammonium phosphate, phthalate, acetate, and formate. Some of these were obtained from commercial sources; others were synthesized locally. Magnesium lignin sulfonate, as obtained, was not a very pure compound and the content of magnesium was very small. While the magnesium ammonium phosphate is not an organic compound, its behavior was so similar to those of this group that it is discussed with them.

Each of the compounds was tested as part of a complete nutrient solution supplied to a given set of cultures, in comparison with controls supplied with Mg as the sulfate. For some series parallel cultures were grown with no Mg in the nutrient solution. All solutions were prepared with distilled water and pure chemicals. The containers were one-gallon size glazed crocks coated on the inside with a waterproof varnish.

Quite early in the conduct of these cultures certain facts were revealed about pH relationships which are worthy of some emphasis. A typical nutrient solution would contain the following salts: $Ca(NO_3)_2$, $(NH_4)_2SO_4$, KCl, KH_2PO_4, $FeSO_4$, small amounts of trace or minor elements, and Mg as the sulfate or as organic compound. In all cultures equivalent amounts of magnesium were supplied. Most inorganic nutrient solutions are slightly acid when first mixed, but tend to become alkaline after plant roots have grown in them for a short time. The pH of such solutions may be regulated to some extent to a constant level by either of two methods: by adding acid, such as dilute sulfuric acid, at intervals to adjust the pH to the desired level, or by adjusting the ratio of the nitrate ion to the ammonium ion as to make a physiological balance of the pH in the nutrient solution.

In these preliminary experiments attempts were made to adjust the pH by adding small amounts of dilute sulfuric acid. It was found that this worked quite well with the solutions containing magnesium as the sulfate. However, with solutions containing organic salts such as magnesium phthalate, the pH remained unchanged even with addition of relatively large amounts of acid, owing to the buffering action of the organic salt. It seemed more advisable to adjust the pH of the inorganic combination of nutrients to a level which would give good growth of plants by adding a small amount of ammonium sulfate along with the calcium nitrate as source of nitrogen to both sets of cultures, and to allow the pH of the solutions containing organic salts of magnesium to remain at a higher level as long as the plants grew in good health and free of chlorosis. This was done for most of these cultures. For example, the pH of the magnesium phthalate nutrient solutions averaged around 7.0 and that of solutions with magnesium acetate about 6.6 as compared with the 4.0 to 4.5 level maintained for the magnesium sulfate solutions. Enough dilute acid was added to the organic salt cultures to keep the phosphate and iron in solution.

Experimental Results: The first two compounds, the thiocyanate and salicylate, were definitely toxic to corn plants, even when used at concentrations providing magnesium at one fourth the amount contained in the sulfate cultures. When the salicylate was added to the other nutrients, it turned the solution a deep wine color. This was found to be due to a reaction with iron as ferrous sulfate. Mold growth was very abundant in the salicylate solutions when allowed to stand for several days.

The dry weight yield data for the plants grown with the other compounds are given in table I. Each compound usually was tested in 10 jars with a pair of plants per jar. Each set of cultures had one or more of the test compounds used in comparison with magnesium sulfate as control and occasionally in

Table I

Effect of Various Organic Carriers of Magnesium on Dry Weight Yields of Corn in Water Cultures—Preliminary Trials

Experiment No.	Treatment	Days age	Mean of dry wt. in gm	Standard error	t
1	$MgSO_4$	33	3.13	0.356	.....
	Mg acetate	33	8.15 *	0.906	5.15
2	$MgSO_4$	33	5.48	0.260	.....
	Mg formate	33	18.61 *	0.219	38.9
3	$MgSO_4$	39	15.00	0.402	.....
	Mg lignin-sulphonate	39	3.30	0.127	27.81
	No Mg	39	2.80	0.149	28.47
4	$MgSO_4$	27	1.10	0.315	.....
	No Mg	27	0.90	0.361	0.417
	Mg phthalate	27	4.50 *	0.456	6.150
	$MgNH_4PO_4$	27	8.80 *	0.256	19.01
5	$MgSO_4$	33	1.80	0.033	.....
	Mg glycero-phosphate	33	1.50	0.026	7.09

* Yields significantly greater than with $MgSO_4$ (1% level).

Exp'ts. 1 and 2—10 pairs of plants per treatment.
Exp'ts. 3 and 4— 7 pairs per treatment.
Exp't. 5 —15 single plants per treatment.

comparison with a set of plants grown with a nutrient solution containing no magnesium. It will be noted in this and subsequent tables that the yields for the different magnesium sulfate plants often differ markedly from one to another. Sometimes this can be attributed partly to differences in age at time of harvest, but more often to the different times of year in which the cultures were grown in the greenhouse. A set of plants usually will grow more slowly in fall and winter than at other times of the year. Some of the variations from one set of cultures to the next may be attributed to initial differences in general size of the plants at the time of starting the cultures. This was particularly true of the apple seedlings.

The yields in table I, together with their corresponding "t" values, indicate that those for the lignin sulfonate, glycerophosphate, and magnesium-free were significantly less than those for the sulfate. The lignin sulfonate, while not toxic, contained so little magnesium as to be of no benefit. The large amounts required to give a comparable amount of the element made it practically impossible to manipulate. The other four compounds, the ammonium phosphate, phthalate, acetate, and formate were distinctly more beneficial to corn plants than magnesium sulfate. There was much more vigorous growth of tops and roots than in the controls.

Cultures with Same pH Level

Materials and Methods: It now became of interest to test the possibility of growing corn or apple seedlings, with the pH levels of all solutions more nearly alike. It seemed possible that the different pH levels, per se, might cause the differences in growth, i.e., that the beneficial effect ascribed to some of the organic salts might be due, not to them, but rather to the higher pH.

In order to test this point, the following experiments were performed:

Several different series of water cultures with corn, as indicated in table IV, were grown in nutrient media with magnesium sulfate as the control. In all sets of cultures two organic salts were tested for comparison.

Table II

Amounts of Nutrient Salts (0.5 M) Contained in 10 Gallons of Solutions for Maintenance of pH Level

Salts used *	$MgSO_4$ cultures	Mg acetate cultures	Mg formate cultures
	ml	*ml*	*ml*
$Ca(NO_3)_2$	30	10	10
$(NH_4)_2SO_4$	10	30	30
$CaCl_2$	0	20	20
KH_2PO_4	30	30	30
$MgSO_4$	20	0	0
Mg acetate	0	20	0
Mg formate	0	0	20

* Iron as $FeSO_4$ and trace elements.

Table III

Relation between NO_3/NH_4 and pH in Nutrient Solutions of Corn Cultures of Experiment 8 of Table IV *

$MgSO_4$ cultures	Mg acetate cultures	Mg formate cultures
2 days	2 days	2 days
30/10 ⟶ pH 4.5	10/30 ⟶ pH 6.5	10/30 ⟶ pH 6.5
Next change of solutions.		
2 days	2 days	2 days
40/0 ⟶ pH 6.5	0/40 ⟶ pH 4.0	0/40 ⟶ pH 4.0
Next change of solutions.		
2 days	2 days	2 days
35/5 ⟶ pH 6.5	5/35 ⟶ pH 4.5	5/35 ⟶ pH 4.5
Next change of solutions.		
2 days	2 days	2 days
30/10 ⟶ pH 5.0	5/35 ⟶ pH 5.0	5/35 ⟶ pH 4.5
Successive changes of solutions with same NO_3/NH_4 ratios as in last line gave the following values till harvest:		
pH 4.5	pH 4.3	pH 4.5
pH 4.5	pH 4.0	pH 4.0

* All solutions equal in pH as first mixed, about 5.0. Ratios represent ml of 0.5 M $Ca(NO_3)$ to ml of 0.5 M $(NH_4)_2SO_4$. Arrows represent shift in pH.

A typical set of nutrient solutions is that given in table II. Each of these groups of solutions would be diluted, mixed, and placed in 10 one-gallon crocks, 30 in all, and two plants grown in each crock.

Some of the organic salts used, notably the magnesium nitrate-urea compound, $Mg(NO_3)_2 \cdot 4CO(NH_4)_2$, and the magnesium anthranilate, had considerable nitrogen in their molecules. This necessitated an adjustment in the amounts of nitrogen in the inorganic solutions to make them equivalent in this respect. Most of the organic salts were weighed out fresh for each change of solutions, which was usually made at 7-day intervals. A magnesium versenate, was prepared, containing a known amount of magnesium, for use in nutrient solutions.

All of the cultures, the yields of which are shown in table IV, were grown with the pH level controlled, i.e., an effort was made to keep it as close as possible to the same level throughout any given series of three variables. This was done by varying the NO_3/NH_4 ratio. Examples of the ways in which differences in this ratio will affect the pH are found in table III.

Experimental Results: An examination of the yields in dry weights from these cultures, as shown in table IV, reveals no better growth for any of the organic salts of magnesium, except one, as compared with the controls with $MgSO_4$. With some the organic cultures were distinctly poorer, an exception being the magnesium fumarate. It is worth noting in this connection that the high amounts of nitrogen in the magnesium nitrate-urea compound and in the magnesium anthranilate made this series of cultures very difficult to grow, and it was also difficult to adjust the amount of nitrogen in the controls to an

TABLE IV

EFFECT OF VARIOUS ORGANIC CARRIERS OF MAGNESIUM ON DRY WEIGHT YIELDS OF CORN IN WATER CULTURE WITH SAME pH LEVEL

EXPERIMENT No.	TREATMENT	AGE IN DAYS	MEAN OF DRY WT. IN GM	STANDARD ERROR	t
6	$MgSO_4$	50	12.3	0.350	....
	Mg nitrate-urea	50	12.0	0.281	0.67
	Mg anthranilate	50	2.9*	0.088	26.08
7	$MgSO_4$	45	36.1	0.302	....
	Mg lactate	45	20.6*	0.325	35.01
	Mg malate	45	33.9*	0.277	5.36
8	$MgSO_4$	42	16.7	0.206	....
	Mg acetate	42	7.3*	0.100	41.06
	Mg formate	42	6.6*	0.046	47.38
9	$MgSO_4$	32	7.4	Not available	
	Mg versene	32	2.5		
	Mg fumarate	32	9.4		
10	$MgSO_4$	46	5.2	0.089	....
	Mg versene	46	2.0*	0.054	29.58
	Mg pyruvate	46	5.1	0.079	0.50

* Yields with $MgSO_4$ significantly greater than this treatment (1% level).

equivalent level. There is one other point that should be considered in connection with this kind of manipulation of the nutrient solutions to maintain a constant pH level. In table III it is shown that the ratio of NO_3 to NH_4 finally established for the organic cultures, as necessary for constant pH, was 5/35, as compared with 35/5 for the inorganic cultures. This means an abnormally high amount of NH_4 is required in the medium containing the organic salt. It has been amply confirmed by other workers that a very high level of NH_4 in a nutrient solution is apt to be toxic. Therefore, it seems inadvisable to follow this procedure for other future cultures, especially in view of results to be considered next.

CULTURES WITH DIFFERENT pH LEVELS

In table V are shown results of several sets of cultures in which the pH was not controlled, i.e., not held at the same level for all nutrient solutions. Here the pH was allowed to remain at a higher level for the organic mixtures. Some of these organic salts had not been tested before. This was true of the benzoate, adipate, sebecate, gluconate, levulinate, and the methyl-, ethyl-, propyl-, and butylphosphates. Except for the propylphosphate these new compounds gave yields significantly less than those from the corresponding sulfate cultures. The other salts tested, the malate, lactate, ammonium phosphate, and the fumarate, promoted better growth than the corresponding controls; in this they were similar to the phthalate, the formate, and acetate used in the preliminary experiments mentioned above.

It seemed desirable at this stage of the investigations to grow apple seedlings in cultures with some of the best of the organic compounds thus far used, in comparison with control cultures supplied with $MgSO_4$. Some previous attempts at growing apple seedlings in nutricultures had revealed several limitations of these methods when applied to such plants: (a) They are not well suited to growing in water culture and do much better in sand culture. (b) A longer time than with corn is required to show effects of treatments and the effects are not so sharply defined. This may be due to a retention of relatively large supplies of magnesium in the wood. This statement is supported by results reported elsewhere (5). (c) Individual variations are apt to be greater among apple seedlings than among corn plants at the close of an experimental period.

In table VI are presented the results from two series of water cultures with apple seedlings and several series in sand culture. The plants were selected for uniformity in size and appearance. For the sand cultures each seedling was grown in a gallon jar with acid-washed quartz sand. After transplanting to the sand, all of the seedlings were supplied only distilled water for several weeks in an attempt to rid the plant body of as much residual magnesium as possible. At the start of a test period each different magnesium compound was supplied in a complete nutri-

TABLE V

EFFECT OF VARIOUS ORGANIC CARRIERS OF MAGNESIUM ON DRY WEIGHT YIELDS OF CORN IN WATER CULTURE WITH DIFFERENT pH LEVELS

EXPERIMENT No.	TREATMENT	AGE IN DAYS	MEAN OF DRY WT. IN GM	STANDARD ERROR	t
11	$MgSO_4$	46	3.6	0.101	....
	Mg malate	46	8.2**	0.114	30.21
	Mg lactate	46	7.0**	0.066	28.97
12	$MgSO_4$	40	4.8	0.102	....
	$MgNH_4PO_4$	40	10.8**	0.278	20.26
	Mg fumarate	40	18.1**	0.204	60.52
13	$MgSO_4$	48	24.4	0.304	....
	Mg benzoate	48	14.7	0.255	24.49
	Mg adipate	48	18.9	0.207	14.98
14	$MgSO_4$	46	7.9	0.154	....
	Mg sebecate	46	2.5	0.028	34.50
	Mg gluconate	46	5.4	0.127	12.53
	Mg levulinate	46	6.5	0.103	7.55
15	$MgSO_4$	46	12.7	0.228	....
	Mg methyl-phosphate	46	13.5*	0.225	2.50
	Mg ethyl-phosphate	46	9.3	0.237	10.34
16	$MgSO_4$	60	12.9	0.204	....
	Mg propyl-phosphate	60	13.8*	0.285	2.42
	Mg butyl-phosphate	60	9.9	0.212	10.20

* Significant (5% level).

** Yields significantly greater than with $MgSO_4$ (1% level).

TABLE VI

EFFECT OF VARIOUS ORGANIC CARRIERS OF MAGNESIUM ON DRY WEIGHT YIELDS OF APPLE SEEDLINGS IN WATER AND SAND CULTURE WITH DIFFERENT pH LEVELS

EXPERIMENT No.	TREATMENT	AGE IN DAYS	MEAN OF DRY WT. IN GM	STANDARD ERROR	t
	Water culture				
17	$MgSO_4$	76	4.10	0.109	....
	Mg acetate	76	3.5	0.139	1.07
18	$MgSO_4$	75	0.37	0.026	....
	Mg lactate	75	1.05*	0.057	18.97
	Mg malate	75	1.66*	0.109	11.54
	Sand culture				
19	$MgSO_4$	140	5.5	0.161	....
	Mg acetate	140	10.1*	0.237	16.07
	No Mg	140	1.4	0.090	22.21
20	$MgSO_4$	150	13.8	0.658	....
	Mg formate	150	26.5*	0.626	13.99
	Mg phthalate	150	22.6*	0.964	7.54
	Mg acetate	150	27.0*	0.594	14.89
21	$MgSO_4$	127	2.4	0.110	....
	Mg malate	127	11.6*	0.507	17.74
	Mg fumarate	127	12.8*	0.760	13.55
22	$MgSO_4$	57	3.4	0.207	....
	Mg lactate	57	5.6*	0.146	8.69
	Mg propyl-phosphate	57	6.1*	0.192	9.56

* Yields significantly greater than with $MgSO_4$ (1% level).

ent solution to 10 plants at weekly intervals. No attempt was made to keep the pH at a uniform level throughout.

The dry weight data given in table VI indicates beneficial effects from all of the organic carriers of magnesium there listed. All produced significantly greater yields than those with $MgSO_4$, except in water cultures with magnesium acetate. The acetate proved better than the sulfate in the sand cultures, however. The dry weight figures were quite indicative of the average relative size and vigor of the plants. Most of those grown with magnesium supplied in organic form were taller, and had more leaves and thicker stems than those with $MgSO_4$. These data were taken, but are not presented here. It is worth noting that the promising results found with magnesium fumarate in previous corn cultures (table IV, expt. 9, and table V, expt. 12) are confirmed in these tests with apple.

As far as evidence of scorch or injury is concerned, there was almost none of it on any of the plants except a slight burning on a few leaves of the sulfate group. This did not resemble typical leaf scorch as seen in the field.

DISCUSSION

Considerable evidence has been presented which shows that certain organic compounds of magnesium produce better growth in corn and apple seedlings than that produced by magnesium sulfate in a complete nutrient solution. This is true when pH of the nutrient solutions is not kept at the same level, but rather the buffering action of the organic compounds is allowed to maintain the pH of the solutions at a level higher than the pH of the magnesium sulfate solutions. The technique of maintaining the pH values of nutrient cultures for magnesium studies constant by adjustment of the NO_3/NH_4 ratio seems valueless for the conduct of such investigations. Further weight to this conclusion is given by the findings of Arnon et al (1) who noted that the pH of an external solution could vary between 4 and 9 and still produce no profound effects on plant absorption of magnesium, potassium, and nitrate. Arnon and Johnson (2) also showed that most plants are not adversely affected by pH variations except at extreme ends of the range, below 4 and above 8.

To suggest reasons for the advantageous effect of these organic carriers compared with that of the sulfate would be to speculate. Concerning this it may be of interest to cite the work of Lott (6) on a magnesium deficiency in grape vines. Treatments to alleviate this included soil and foliar applications of magnesium acetate. Subsequent soil analyses showed larger amounts of extractable magnesium in the acetate-treated soil than in the corresponding sulfate-treated one. The author suggests a mechanism occurring in the soil for making the Mg readily available to plants by microbial decomposition of the acetate and conversion of magnesium into the carbonate by replacement of the acetate radical in the root zone.

SUMMARY

Several organic carriers of magnesium in comparison with magnesium sulfate were tested for their suitability for alleviation of apple leaf scorch. Corn and apple seedlings were grown in water and sand cultures in which each of these several compounds in turn were a part of a complete nutrient solution.

1. Apple seedlings grew much better in sand culture than in water culture and the results in the former are more reliable.

2. The buffering effect of the organic carriers kept the pH of the nutrient solutions at higher levels than that in those solutions containing magnesium sulfate. When all solutions were maintained at the same lower level by adjustment of the NO_3/NH_4 ratio, adverse growth effects were produced with most of the organic compounds tested.

3. When the pH of those solutions containing organic carriers was allowed to remain high there was markedly better growth with many of them than with the magnesium sulfate controls. Of the compounds tested two were toxic: magnesium thiocyanate and salicylate. Fourteen gave no benefit: lignin sulphonate, glycerophosphate, versene, nitrate-urea, anthranilate, pyruvate, benzoate, adipate, sebecate, gluconate, levulinate, methyl-, ethyl-, and butyl-phosphates. Seven were distinctly more beneficial than sulfate to both corn and apple growth. These were: acetate, formate, phthalate, ammonium phos-

phate, malate, lactate, and fumarate. Propyl phosphate was beneficial for apple but not for corn.

The assistance to these investigations of Dr. Joseph Seiberlich, of the New Hampshire Engineering Experiment Station, in synthesis of certain of the organic compounds of magnesium used, of Mr. G. P. Percival, in pH tests of solutions, and of Mr. Howard Moulton for the care of some of the cultures, is gratefully acknowledged.

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TRANSLOCATION OF RADIOACTIVE ISOTOPES FROM VARIOUS REGIONS OF ROOTS OF BARLEY SEEDLINGS[1,2]

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Although much attention has been given to the uptake of minerals by roots it is not known from which regions of the root most of the translocation of minerals to the shoot occurs. The fact that minerals are accumulated freely in a certain region of the root does not prove that they are translocated out of that region equally freely. In fact it might be expected that those regions which accumulate minerals most rapidly would release them most slowly. The evidence on this problem is limited and contradictory. Prevot and Steward (18) found the greatest accumulation of bromide ion in the apical region of excised barley roots, and Steward et al (22) found a similar graduation of accumulation of rubidium and bromide in attached barley roots. The latter investigators also found that attached barley roots which were transferred to water after 24 hours in rubidium bromide solution lost these ions most rapidly from the apical region in which accumulation had been greatest. Lundegardh (14), on the other hand, found by analysis of the exuding sap that the greatest translocation of nitrate occurred from the basal region of wheat roots. He suggested that nitrate was rapidly absorbed by all regions, but more nitrate was reduced in the younger regions, leaving less available for translocation from near the apex.

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Some of our previous work (12) indicated that heavy accumulation of radioactive phosphorus occurs several centimeters behind the root tip and that it is readily translocated from this region to other parts of the plant. A series of experiments was therefore performed to study the relative amounts of several radioactive isotopes absorbed at various distances behind the root tip and translocated to other parts of the seedling. Use of radioactive isotopes made it possible to observe movement of very small amounts of material over short periods of time with relative ease.

Methods

Isotopes Used: The radioactive isotopes used were P^{32}, Rb^{86}, I^{131}, S^{35}, Ca^{45}, and Sr^{90}. Monovalent cations and anions are commonly used in studies of mineral accumulation because it is believed that they do not generally enter into organic compounds. As a result the study of accumulation is not complicated by metabolic removal of the ions from the vacuolar solution. Rb^{86} and I^{131} were selected as representative monovalent ions. They probably remain in the vacuolar sap in ionic form. The absorption of Rb appears to occur on the same sites as absorption of K, and iodide appears to be absorbed at the same sites as bromides and chlorides (5, 6). Also these elements, if initially present in the plant at all, occur only in trace amounts; hence absorption of the radioactive isotope is a good measure of absorption of the element. This is not necessarily true of P^{32}, Ca^{45}, or S^{35} because these isotopes could conceivably

enter the plant in exchange for stable isotopes of the respective elements initially present in the plants. The rubidium and iodide ions could not enter in exchange for stable isotopes of the same elements, although they might enter in exchange for ions of other elements already present (5, 6).

The isotopes were supplied to the plants in full strength Hoagland's solution because it was believed that if the isotopes were supplied as tracers in the usual concentration of stable isotopes they would give a better indication of mineral absorption and translocation than if supplied in extremely dilute, carrier-free solution where surface precipitation and exchange are relatively more important.

The basic nutrient solution consisted of 5 millimoles of KNO_3, 5 millimoles of $Ca(NO_3)_2$, 2 millimoles of $MgSO_4$, and 1 millimole of KH_2PO_4 per l. Sufficient quantities of the various isotopes were added to this base solution to give an activity of 2 to 3 mc per l. The isotopes were added in the following chemical forms: P^{32} as KH_2PO_4, Rb^{86} as RbCl, I^{131} as KI, S^{35} as $MgSO_4$, Ca^{45} as $CaCl_2$, and Sr^{90} as $SrCl_2$. One millimole of stable KI was added per l of the I^{131} solution and the Rb^{86} solution contained about 18 millimoles of carrier RbCl per l. No inert carrier was added to the Sr^{90} solution.

Culture of Barley Seedlings: The Sacramento variety of barley was used in these experiments. The seedlings were grown by a method modified from that described by Machlis (15). The seeds were soaked in water for 15 minutes, the hulls removed, and the moist seeds placed in a flask where they were kept moist for a day by repeated rinsing with water, and at the same time they were well aerated. The germinating seeds were then transferred to glass grids so arranged in 700-ml cylinders that the coleorhizas were in contact with dilute nutrient solution. The dilute nutrient solution consisted of the basic nutrient solution described earlier diluted with 1000 volumes of water. The cylinders were painted black on the outside to reduce illumination of the roots. The nutrient solution was aerated continuously and was changed about 15 times a day by slow gravity flow from a reservoir. The seedlings were grown either in a laboratory window or in a constant temperature room at 24 to 26° C with 12 hours of illumination daily from fluorescent and mazda lamps. The seedlings developed long, uniform, unbranched roots in 5 days after the initial soaking of the seeds.

In our earlier experiments on absorption of P^{32} (12) many irregularities in absorption were observed which appeared to be related to variations in composition and aeration of the nutrient solution. Growth of seedlings in flowing nutrient solution greatly reduced this variation. Use of a dilute nutrient solution produced seedlings with a low salt content, yet avoided the abnormal growth sometimes observed in distilled water.

Method of Supplying Isotopes: An absorption cell (fig 1) was developed for supplying the isotope solution to limited regions of individual roots under normal conditions for mineral absorption and growth. These cells were suggested by the circulation vessels of Lundegardh (13) and the potometers of Hayward, Blair and Skaling (8). The glass portion consisted of a tube 10 mm in diameter with a side arm. The tube was drawn out and bent around so that the narrow end faced the side arm. This end and the side arm were connected by pure gum rubber tubing, 3 mm in diameter, designed to hold a portion of the root. A hole was burned through both walls of the rubber tubing by using a hot nichrome wire of the same diameter as the roots. The holes were then connected

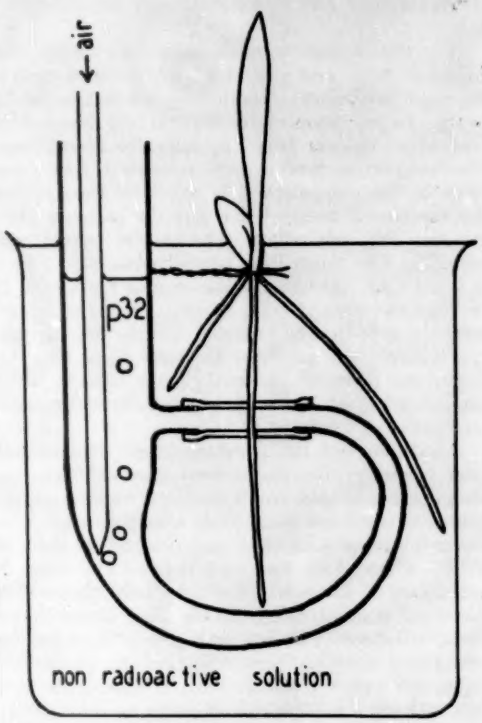

Fig. 1. Diagram of the absorption cell used in supplying radioactive isotopes to localized regions of roots. A segment of the selected root 3 mm in length was inclosed in soft rubber tubing through which the isotopes were circulated while the other roots were immersed in flowing non-radioactive nutrient solution.

by a slit. Any portion of the root could be fitted into this tubing. Warm, almost liquid lanolin was used to seal the junction between root and rubber. A nichrome wire holder held the grain of the seedling in place. The solution containing the radioactive isotope was then placed in the absorption cell, and the entire apparatus was immersed in the dilute flowing solution. Gentle tapping usually dislodged air bubbles trapped inside the rubber tubing.

Air bubbling through the solution in the large arm of the absorption cell both aerated and circulated the solution. The level of the isotope solution was usually kept 0.5 inch below the level of the external cul-

ture solution so that if leaks developed, the flow would be inward where it could be detected by a rise in the level of the solution. In actual practice leakage occurred in less than 10 % of the experiments. The external culture solution also was aerated and was changed at the rate of 10 volumes per hour to keep contamination by leakage or leaching from the roots to a minimum. Contamination of the external solution was low as indicated by the small amount of radioactivity in control plants which were placed in the solution with the non-treated roots of plants supplied with isotopes through the absorption cells.

The experiments with absorption cells were conducted at 25° C and were of 6 hours duration excepting some preliminary experiments which ran for 12 hours. In repetition of the experiments plants were selected at random from the same lot of seedlings. The fact that roots often grew as much as 3 or 4 mm while in the absorption cells indicated that neither the mechanical manipulation nor the radiation during the relatively short experimental period was harmful. The plants were treated when they were 5 to 6 days old. At this age the seminal roots were 10 cm long and were growing rapidly. As roots become older the growth rate decreases and the different tissues mature more nearly to the apex (7, p. 491, 10). Treatment therefore occurred at the time of maximum longitudinal separation of the different anatomical regions of the roots.

Measurement of Translocation: Immediately after treatment the plants were removed from the absorption cells and rinsed in three changes of the diluted nutrient solution. They were then placed between layers of glass cloth and dried in an oven at 95° C. Glass cloth was used because the roots do not adhere to it. Autoradiographs were prepared in the usual manner, using Kodak Blue Brand X-ray film. Goodyear Pliofilm was placed between the plants and the film, except for the low energy isotopes, C^{45} and S^{35}, which were placed in direct contact with the film. Plants containing no radioactivity were exposed at the same time to detect the effect on the film of any chemical compound found in barley plants (4). No such effects were noted.

After preparation of the autoradiographs the plants were cut up and counted for radioactivity. The portions of the treated root within 2 mm of the region of isotope supply were counted as one sample. The apical and basal portions of the treated root were counted separately as were the non-treated roots, grain, and leaves from each plant. Roots from the control plants were also counted. The counts were corrected for background radiation and for contamination of the external solution as determined by the control plants.

Plants supplied with P^{32}, Rb^{86}, I^{131}, and Sr^{90} were counted directly with a thin-end-window Geiger tube and scaler. Self absorption of radiation from these isotopes is not great with dry organic material of small diameter such as these barley roots and was presumed to be quite uniform since the roots increase very little in diameter over their entire length. Material containing Ca^{45} was ashed in an electric furnace at 500° C, and the dry powdery ash was cemented to the dish with a drop of dilute collodion. Material containing S^{35} was digested directly in glass counting dishes or in 1-inch watch glasses. The roots were covered with about 0.5 ml of concentrated HNO_3 for 12 hours after which several drops of H_2O_2 were added. After about 3 more hours the samples were slowly evaporated to dryness under infra-red lamps. The Ca^{45} and S^{35} samples were counted in a windowless flow counter.

Results and Discussion

Figure 2 shows photographs of autoradiographs obtained in a preliminary experiment using 5-day-old plants and supplying the P^{32} for 12 hours instead of the usual 6 hours. On these autoradiographs the amount of P^{32} present is approximately proportional to the blackness and width of the image. In plant A the isotope was supplied about 5 cm from the root

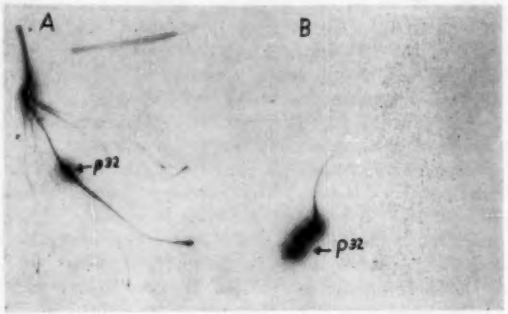

Fig. 2. Translocation of P^{32} supplied to roots of barley seedlings at the points indicated by the arrows. When supplied 5 cm behind the tip (left) it was translocated throughout the seedling, but little translocation occurred when it was supplied to the root tip (right).

apex. Large quantities were translocated to the grain and leaf (the upper portion of the leaf lies to the right of the grain) and to the tips of the non-treated roots as well as to the tip of the treated root. Plant B was supplied with P^{32} at the root apex. A somewhat larger quantity of P^{32} was absorbed than by plant A, but so little was translocated upward that it was scarcely detectible in the other parts of this seedling. The slightly greater P^{32} absorption of plant B may have been due to the fact that more surface was exposed, since the tip of this root grew several mm while it was in the absorption cell. Autoradiographs of later replications were similar to the one pictured.

In further experiments four general regions were selected for the placement of the absorption cells. The first was the terminal 3-mm portion of the root which comprises the meristem and some elongating cells. The second region was about 6 to 9 mm from the apex, representing the region in which the protoxylem may be mature. In the third region, 20 to 30 mm from the apex, the metaxylem is mature and the

TABLE I

TRANSLOCATION OF ISOTOPES FROM VARIOUS REGIONS OF THE ROOT. VALUES ARE COUNTS PER SECOND AND INDICATE THE AMOUNT OF ISOTOPE PRESENT IN THE REGION TO WHICH IT WAS SUPPLIED AND THE AMOUNT TRANSLOCATED TO OTHER PARTS OF THE PLANT

ISOTOPE	PHOSPHORUS[32]				RUBIDIUM[86]			
NO. OF REPLICATIONS	3	3	3	3	2	4	3	4
DISTANCE OF SUPPLY FROM ROOT TIP IN MM	0–5	6–9	28–31	82–85	0–4	6–9	20–23	58–61
Apical region of treated root	.....	.....	13.99	36.36	....	....	....	14.47
Treated region *	166.48	146.92	79.84	65.60	21.28	40.44	42.85	23.12
Base, treated root	.39	2.96	6.5	.82	.44	5.25	1.88	.10
Non-treated roots	.93	4.35	14.86	12.75	.49	.82	3.98	2.50
Leaf	.72	5.45	25.09	19.22	.00	.37	.94	.96
Grain	.24	.79	2.66	2.37	.02	.31	.56	.34
Total translocated	2.28	13.54	49.11	34.14	.94	6.75	7.36	3.90
Total absorbed	168.76	159.97	142.94	137.10	22.22	47.18	50.21	41.48
% translocated	1.35	8.47	34.4	24.9	4.24	14.3	14.7	9.4
Standard deviation	1.03	4.70	15.8	5.4	4.5	6.0	4.4	6.4
ISOTOPE	IODINE[131]				SULFUR[35]			
NO. OF REPLICATIONS	3	3	3	3	2	2	2	2
DISTANCE OF SUPPLY FROM ROOT TIP IN MM	0–4	7–10	25–28	69–72	0–4	7–10	23–26	59–62
Apical region of treated root	.....	.....	....	17.23	....	....	....	8.85
Treated region *	101.20	103.76	81.10	44.89	32.02	21.91	14.75	18.76
Base, treated root	.59	26.16	16.57	.99	.10	.26	.23	.25
Non-treated roots	.28	7.99	9.07	10.23	.18	.40	1.03	1.62
Leaf	.18	3.58	5.03	4.21	.22	.08	.09	.18
Grain	.00	3.27	2.32	2.83	.11	.46	.62	.74
Total translocated	1.05	41.00	32.99	18.26	.61	1.20	1.97	2.79
Total absorbed	102.30	144.80	114.12	80.41	36.63	23.11	16.72	30.40
% translocated	1.03	28.3	28.9	22.7	1.7	5.2	11.8	9.2
Standard deviation	0.7	18.3	13.5	2.4	.9	2.3	4.3	3.0

* The treated region has a 2 cm segment including the portion of the treated root enclosed in the absorption cell.

endodermis is developed but passage cells remain. The fourth region was between 55 and 70 mm from the apex where the endodermis forms a complete cylinder, uninterrupted except by lateral root primordia.

Results of counts of the various parts of the seedlings are summarized in tables I and II. There was relatively little translocation of any of the isotopes from the terminal 4 mm of the root and maximum translocation generally occurred from the region about 10 to 30 mm above the apex. Upward translocation from basal portions of roots was generally less than from the middle, but much greater than from the apex.

TABLE II

SUMMARY OF DATA ON TRANSLOCATION OF RADIOACTIVE ISOTOPES FROM VARIOUS REGIONS OF ROOTS. EACH VALUE REPRESENTS THE AMOUNT TRANSLOCATED AS A PERCENTAGE OF THE TOTAL AMOUNT ABSORBED AND IS THE AVERAGE OF TWO TO FOUR REPLICATIONS

APPROXIMATE DISTANCE FROM ROOT TIP AT WHICH ISOTOPE WAS SUPPLIED	% OF ISOTOPE TRANSLOCATED UPWARD			
	P^{32}	Rb^{86}	I^{131}	S^{35}
0– 4 mm	1.3	4.2	1.0	1.7
7–10 mm	8.5	14.3	28.3	5.2
27–30 mm	34.4	14.7	28.9	11.8
57–60 mm	24.9	9.4	22.7	9.2

In some plants large quantities of isotope accumulated in the basal regions of the treated roots, presumably because it was absorbed from the xylem stream. This undoubtedly reduced the amount reaching the shoot and untreated roots. For example, when I^{131} or Rb^{86} was supplied about 8 mm from the apex, larger quantities of these isotopes were found in the basal portions of the treated root than in the grain, leaf, or untreated roots. The amount of upward translocation was defined as the quantity of an isotope that had moved more than 2 cm up the treated root from the point of supply and includes all that found in the grain, leaf, and non-treated roots. Downward translocation in the treated root was defined as the quantity of isotope which moved more than 2 cm toward the apex from the point of supply.

The length of the treated root had little effect on the amount translocated upward. In several preliminary experiments roots of different lengths were selected and the absorption cell was placed at a standard distance from the grain while its distance from the root apex depended on the length of the root. Examination of autoradiographs revealed that

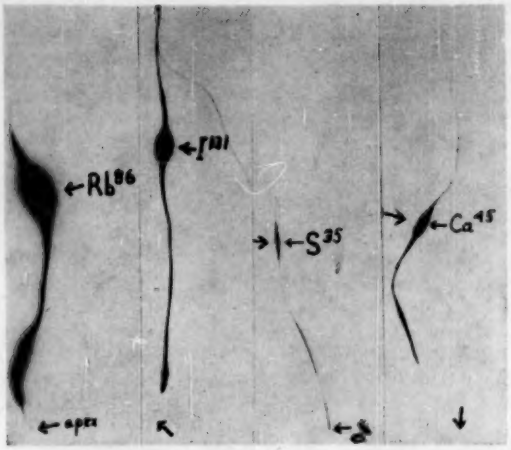

FIG. 3. Autoradiographs showing, from left to right, the pattern of downward translocation of R^{86}, I^{131}, S^{35}, and Ca^{45} in roots of barley seedlings. The upper arrows indicate the points of supply, the lower arrows indicate the location of the root tips.

the amount of upward translocation was related to the distance of the supply from the root apex rather than the distance to the grain.

Most of the isotopes were readily translocated to the leaves as well as to the non-treated roots. Relatively less reached the grain and almost all of the radioactivity recorded for the grain was in the stem-root junction which usually was not separated from the grain before counting. In a few cases the storage tissue of the grain was counted separately and found to contain very little radioactivity. P^{32} and I^{131} were most readily translocated while smaller proportions of the absorbed Rb^{86} and S^{35} were translocated to the leaves and non-treated roots. The quantities of Ca^{45} translocated were small and difficult to detect with certainty above the slight contamination of the external solution. It appeared, however, that Ca^{45}, like the other isotopes studied, was more rapidly translocated from the mature regions of the roots than from the root tips. In the one experiment with Sr^{90} no measurable translocation occurred more than 20 mm above or below the point of supply.

All of the isotopes moved downward in the treated roots, but the pattern varied somewhat for the different isotopes (fig 3). P^{32} and S^{35} moved all the way to the root tip and usually accumulated there. I^{131} and the cations generally did not move to the root tip but stopped, often rather abruptly, at a distance of 3 to 15 mm from the apex. Often the isotopes accumulated in this region. No explanation of this effect is apparent.

The results of this experiment differ from those obtained by Steward, Prevot, and Harrison (22) by another method. They reported that the terminal cm of roots of intact barley plants absorbed RbBr most rapidly and also lost this salt most rapidly by translocation to other parts of the seedling when the roots were transferred to distilled water. They also reported that their roots ceased elongation and the tips became hypertrophied during the experiments which lasted 66 to 72 hours. It is quite possible that during this period of time xylem vessels were matured almost to the tips of the roots, resulting in increased translocation away from the tips. Some Rb apparently was also lost to the external solution in their experiments; hence it is impossible to determine the relative importance of translocation and loss by leaching in removal of salt from any particular region of the root.

MOVEMENT OF ISOTOPES THROUGH A KILLED SEGMENT OF THE ROOT: A few experiments were conducted to determine the tissue in which the isotopes move through the roots. A segment of the intact root was killed by directing a jet of steam on it for 20 seconds. Since upward movement of the isotopes was reduced by only about 30 %, it seemed likely that the isotopes moved upward in the xylem. Steaming either above or below the region of P^{32} entry reduced upward movement by 30 %.

The effect of steam girdling on downward translocation was more variable. In these experiments roots were steamed about 1 cm below (toward the apex) the point of P^{32} application (fig 4). Steaming almost completely prevented all movement of P^{32} downward into the apical cm of the root. Even steam girdling above the region of P^{32} entry reduced movement into the terminal centimeter by over 50 %.

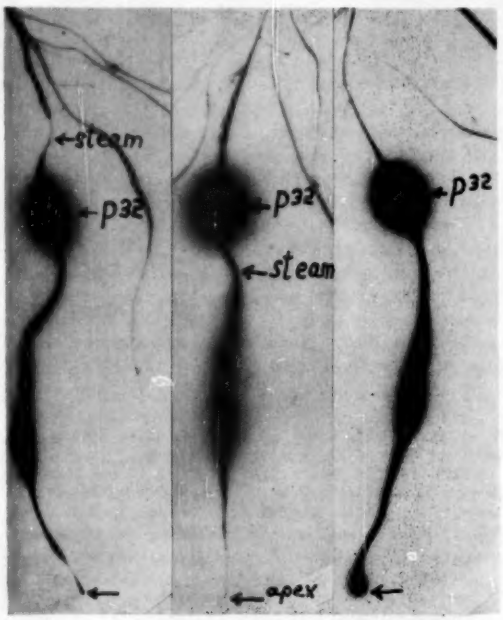

FIG. 4. Autoradiographs showing the pattern of downward translocation of P^{32} in roots which were girdled by steaming above the point of isotope supply (left), below the point of supply (center), with an untreated control root (right).

It is quite possible that downward translocation of P occurs in association with organic compounds. Girdling between the source of organic compounds in the grain and the region of P supply would reduce the movement of organic compounds past this region and, as a result, might easily reduce downward translocation of P^{32}. A "remote" effect on translocation has been reported by Böhning, Swanson, and Linck (3). They found that chilling the hypocotyl of bean plants apparently reduced translocation of carbohydrates from leaves to the stem apex.

Four complete steam girdling experiments were carried out with P^{32} and one each with S^{35}, Rb^{86}, and I^{131}. In all of these, transfer into the apical cm of the root was negligible, but in over half of the experiments there was as much downward transfer of the isotopes past the steamed region as in the roots of unsteamed control plants. It is not known how, or through what tissue this downward movement occurred. Since the distance was only a few cm, it may have diffused downward. The steamed segment was about 8 mm in length, and the killing might have increased the permeability of the cells.

The Role of the Endodermis: It has been suggested by several investigators that the endodermis presents a serious barrier to the entrance of solutes (1, 17, 18, 20). Examination of cross sections of 6-day-old barley roots showed that the inner walls of all endodermal cells, including passage cells, were thickened at distances of more than 5 cm above the root apex. Prevot and Steward (18) found the same situation in the roots they studied. Nevertheless, the results of these experiments show that the endodermis is not an effective barrier to the inward movement of various ions. Neither does it prevent the entrance of water, because considerable water absorption has been observed at distances of several cm above the apex (8, 14, 19, 21).

Reduced Translocation from the Root Apex: There are several possible explanations for the relatively small amount of translocation from the apical portion of the root. Since growing cells can accumulate large amounts of mineral salts, it is possible that all of the absorbed isotope is held by the growing cells. The ability of meristematic cells to accumulate P^{32} and S^{35} from other parts of the plant is illustrated in figures 3c and 4c. In these experiments the root tips contained more P^{32} and S^{35} than the regions just back of the tips. Others have reported on the ability of meristematic and growing tissues to accumulate P^{32} from the rest of the plant (2, 16).

Lundegardh (14) has presented data which substantiate this possibility. In studies on exudation from segments of wheat roots he found larger quantities of nitrate in the exudate from basal segments than from apical segments. He suggested that this occurred because the nitrate, as potassium nitrate, was absorbed as rapidly in the apical as in the basal segments of the roots but the nitrate was more rapidly reduced in the younger parts of the root and, therefore, less of it was available to be translocated from that region. It is probable that phosphate and sulfate are also bound in organic compounds more rapidly near the root tip, and that for this reason smaller quantities of these salts are available for translocation from the tip. Our experiments with isotopes unfortunately, do not distinguish between organic and inorganic combinations of the radioactive isotopes.

The incorporation of sulfates and phosphates in organic compounds might partially explain the failure of these salts to move out of the root apex. Other explanations, however, must be advanced for the retention of iodide and rubidium ions in the apex because they are believed to remain in ionic form in the cells. In this connection it was noticed that usually the latter two isotopes did not move completely to the apex in the absorption cell studies (see fig 3). In contrast, P^{32} and S^{35} invariably became more concentrated in the apex of the root than in immediately adjacent regions (figs 3c, 4c) demonstrating that some substances can move down to the apex. Since the apical region does not readily absorb iodide and rubidium ions from the rest of the root, it is unlikely that it would prevent upward movement of these ions.

A more probable explanation for the very slight translocation from the apex is based on the observation that the xylem is not matured completely to the root tip. Heimsch (10) has reported that in unbranched, growing roots of Sacramento variety of barley, the same variety used in these experiments, the protoxylem matures 0.5 to 1.0 cm from the apex and the metaxylem matures more than 0.8 to 2.0 cm from the apex. Since the roots used in these experiments were young and growing rapidly, it is probable that the xylem was usually not mature within 0.5 to 1.0 cm of the apex. In older or more slowly growing roots, the xylem is differentiated nearer the apex (10, 7). Below the region of mature vessels upward translocation would appear to be by relatively slow diffusion.

This explanation assumes that upward translocation of mineral salts is mainly in the xylem, an assumption for which there is considerable experimental evidence (2, 23) and which is supported by the steam girdling experiments reported earlier in this paper. Although direct experimental evidence is lacking, the absence of mature xylem vessels in the apical region could account adequately for the insignificant translocation from this region.

Such an explanation of the limited translocation of minerals from the meristematic region agrees with what is known about water intake. Most investigators agree that little water is absorbed through the root tips and maximum water intake occurs through the region of the root where the xylem is maturing (8, 9, 21), which also is the region from which maximum translocation of the various ions occurred in our experiments. As the upward translocation of ions appears to occur in the xylem it is not surprising that there is a close correlation between the regions of maximum water and mineral absorption. Hylmo (11) has recently presented convincing evidence of increased absorption of $CaCl_2$ by pea plants accompanying increased absorption of water.

In conclusion it appears that although the meristematic regions of roots accumulate large amounts of minerals, relatively little is translocated from this region to other parts of the plant. The greatest amount of translocation of minerals to the shoot apparently occurs from a region several cm behind the root tip where the xylem is fully matured.

Summary

The translocation of radioactive isotopes from various regions of the root was studied in attached roots of barley seedlings. The isotopes used were P^{32}, S^{35}, Ca^{45}, Rb^{86}, I^{131}, and Sr^{90}. Absorption cells were developed by which the isotope solution could be supplied to any 3 mm region of a root while the remainder of that root and the other roots of the seedling were kept in nonradioactive nutrient solution. The absorption cells were attached at the tip about 10 mm, 20 to 30 mm, and 55 to 70 mm behind the tip.

Although the tips absorbed the various ions freely, very little upward translocation of any of them occurred from the terminal 5 mm region of the roots. Greatest translocation occurred from the region 30 mm back of the root tip. Translocation from the region more than 50 mm above the tip was somewhat lower, but much greater than from the apical region. Upward translocation of Ca and Sr was very limited.

All isotopes moved downward in the treated roots. P^{32} and S^{35} moved down to the tips of the roots and accumulated there. Rb^{86}, Ca^{45}, Sr^{90}, and I^{131} moved down also, but stopped several mm short of the root tips.

Killing a narrow segment of root tissue with steam reduced movement of P^{32} both upward and downward, whether the steamed segment was above or below the point where phosphorus was supplied. Killing tissue in one part of the root seems to affect translocation elsewhere in the root as well as through the dead tissue.

The results of these experiments suggest that most translocation of minerals to the shoots occurs from a region of the roots several cm behind the root tips. This seems to coincide approximately with the region of maximum water absorption.

Part of the work was done while the senior author was at North Carolina State College of Agriculture and Engineering. Grateful acknowledgement is made of the cooperation of Drs. D. B. Anderson, H. T. Scofield, and N. S. Hale of that institution in providing space and equipment, and for their helpful suggestions. Mr. Charles Averre assisted in the laboratory work.

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EFFECT OF DIFFERENTIAL NITROGEN, POTASSIUM, AND MAGNESIUM SUPPLY TO YOUNG VALENCIA ORANGE TREES IN SAND CULTURE ON MINERAL COMPOSITION ESPECIALLY OF LEAVES AND FIBROUS ROOTS[1]

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Young, budded Valencia orange trees were grown for several years in outdoor sand cultures with three levels each of nitrogen, potassium, and magnesium in factorial combinations. Total growth and fruitfulness were greatly dependent on the nitrogen level but relatively independent of the two base elements over a very wide range of supply. These responses and those pertaining to fruit quality were recently described in detail (18).

The nutritional status of the trees was evaluated by yearly collections of leaf samples. At the termination of the experiment in January, 1951, samples of leaves, fibrous roots, and woody structures were taken. The present report describes some of the results of mineral analyses made on these tissues. About 5,000 individual determinations of elements were made on over 900 samples. Only mean values are presented in the tables, for illustrative purposes. They represent the main trends and relations found.

MATERIALS AND METHODS

Details of culture method have been described elsewhere (18). The trees were grown under differential treatment for slightly over three and one half years and satisfactory growth and fruiting resulted where the combinations of nutrients were favorable. The levels of K and Mg were changed somewhat during the second year to alleviate excessive Mg deficiency. During most of the culture period the following concentrations, in ppm, of the three elements were used: N 30, 80 and 200; K 8, 32 and 180; and Mg 12, 24 and 72. These are referred to hereafter as low, intermediate, and high. The N was supplied as 80 % nitrate and 20 % ammoniacal. The other essential elements were supplied in constant amounts as previously described (18).

The first leaf samples were taken in September, 1947, about 3.5 months after the start of the experiment. Subsequent leaf samples were taken on dates given in table I. All leaf samples were detergent-washed (19) except the final collections, which were quite bulky, since they contained all the leaves from the 1-tree plots. The fibrous root samples were not washed because of the possibility of altering their base composition through exchange reactions but they were carefully rinsed in water and most adhering sand particles were removed by jarring the dried samples before grinding. The fact that the sand medium was continuously acidic (pH 4.5 to 5.0) is thought to have prevented most compounds from accumulating on the root surfaces as precipitates. Iron determinations for the root tissues, however, are probably meaningless since the roots penetrated the asphalt paint and were partially in contact with the iron containers. Woody samples (including bark) of trunk, limbs, and large roots were also taken.

Most of the determinations of macro-nutrient elements were made by rapid chemical methods as previously described (16). Some were made spectrographically; however, these will not be singled out since the agreement between the two methods has nearly always been satisfactory. All micro-elements, except Zn, were determined spectrographically. Zinc was determined colorimetrically by standard carbamate procedure. Analysis of variance was made by the appropriate methods outlined by Cochran and Cox (6) for a $3\times3\times3$ factorial design with 4 replications per treatment.

RESULTS AND DISCUSSION

EQUILIBRIUM OF LEAF COMPOSITION WITH TREATMENT: Interpretations based on tissue analysis are

TABLE I

MEAN COMPOSITION OF VALENCIA ORANGE LEAF SAMPLES TAKEN AT VARIOUS TIMES AFTER THE START OF DIFFERENTIAL NUTRIENT FEEDING ON MAY 25, 1947

DATE	9-4-47	7-11-48	10-20-49	6-8-50	1-18-51
LEAF AGE (MONTHS)	3-4	4-5	4-5	3-4	6-18
NO. LEAVES	12	40	30	48	All
Treatment	*Percent N in leaf dry matter*				
Low N	2.21	2.02	1.97	2.08	2.08
Intermediate N	2.37	2.51	2.53	2.16	2.33
High N	3.07	3.50	3.08	2.73	2.76
L.S.D. @ 5 %	0.08	0.07	0.09	0.09	0.08
L.S.D. @ 1 %	0.11	0.09	0.12	0.12	0.11
C.V.	6.7	5.6	7.8	8.9	7.1
	Percent K in leaf dry matter				
Low K	0.91	1.44	1.09	1.04	0.83
Intermediate K	1.31	1.94	1.52	1.44	1.12
High K	1.83	2.92	2.38	2.49	1.81
L.S.D. @ 5 %	0.15	0.09	0.10	0.16	0.12
L.S.D. @ 1 %	0.20	0.12	0.13	0.21	0.16
C.V.	24.0	8.8	12.7	19.9	20.6
	Percent Mg in leaf dry matter				
Low Mg	0.34	0.06	0.19	0.15	0.18
Intermediate Mg	0.35	0.10	0.24	0.23	0.26
High Mg	0.39	0.22	0.39	0.39	0.39
L.S.D. @ 5 %	0.024	0.013	0.027	0.020	0.014
L.S.D. @ 1 %	0.032	0.017	0.035	0.026	0.019
C.V.	14.3	21.7	20.9	16.3	11.0

L.S.D.—Least significant difference between means at odds indicated.

C.V.—Coefficient of variation = $\sqrt{\frac{\text{Rem. variance}}{\text{mean}}} \times 100$.

The value represents the standard error of a sample of the size given from a single plot, expressed as percentage of the mean.

[1] Received October 28, 1953.

strengthened considerably if it is known that the plant is in equilibrium with the imposed nutritional treatment. It is also important to know approximately how much time is required to approach the equilibrium point with the particular plant and under specified experimental conditions. No such information is available for citrus. The data in table I show some results from repeated samplings of the 108 trees in the experiment.

The first samples, taken 3.5 months after the differential treatments were begun, showed approximately the same spread in N and K concentrations as was found in all later collections. Interactions (discussed later) between these two elements were also evident in the first sampling. The high N values for the last two sampling dates were somewhat lower than previously, probably because of the relatively large crops of fruit then present.

In contrast to these two elements, leaf Mg concentrations showed only a small difference between treatment levels after 3.5 months. A large spread in the values was found after 13 months, but all values were lower than desired, indicating an inadequate supply of this element. In the fall of 1948 the Mg levels were increased and the K levels decreased. These shifts raised the leaf levels of Mg found in the fall of 1949 and an equilibrium was reached as evidenced by the similar values found in subsequent analyses.

These data demonstrate that a citrus tree does not reach a balance with all nutrients with equal speed even when all are supplied as soluble ions. The time required to reach equilibrium under orchard conditions after a change in fertilizer practice would probably be much longer than indicated here for base elements because of exchange reactions and other soil factors.

The coefficients of variation indicate that sample size was adequate to reflect the nutritional status of these trees for these elements but they also indicate considerable uncontrolled variability or error. Part of this variability is due to analytical error and part to inherent difference in individual tree responses shown by young trees used as 1-tree plots. Estimates of variance due to analytical techniques have been published (16), but this source accounts for only about one fourth of the error indicated here.

Comparison of Leaf and Fibrous Root Anal-

Table II

Mean Mineral Composition (Portion of Dry Matter) of Leaves and Fibrous Roots of Valencia Orange Trees Grown with Different Levels of Nitrogen, Potassium, and Magnesium*

Treatment	N		P		K		Ca		Mg		Na	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
	%		%		%		%		%		%	
Low N	2.08	1.41	0.334	0.212	2.02	1.16	2.56	1.84	0.289	0.287	0.182	0.042
Intermediate N	2.33	1.46	0.162	0.128	1.11	0.98	3.24	1.92	0.269	0.401	0.135	0.087
High N	2.76	1.85	0.129	0.100	0.62	0.81	3.93	2.22	0.267	0.384	0.118	0.170
Low K	2.44	1.58	0.201	0.144	0.83	0.60	3.72	2.00	0.302	0.470	0.143	0.132
Intermediate K	2.39	1.57	0.216	0.149	1.12	0.82	3.43	1.97	0.273	0.405	0.141	0.119
High K	2.35	1.57	0.208	0.147	1.81	1.53	2.58	2.01	0.250	0.197	0.152	0.048
Low Mg	2.42	1.54	0.212	0.145	1.34	1.03	3.29	1.89	0.182	0.314	0.153	0.122
Intermediate Mg	2.38	1.54	0.219	0.146	1.29	0.94	3.28	2.08	0.257	0.349	0.144	0.092
High Mg	2.37	1.64	0.194	0.149	1.12	0.98	3.16	2.01	0.387	0.408	0.140	0.085
L.S.D. @ 5%	0.08	0.06	0.023	0.008	0.122	0.08	0.15	0.26	0.014	0.038	0.016	0.015
L.S.D. @ 1%	0.11	0.09	0.030	0.011	0.161	0.10	0.20	0.34	0.019	0.051	0.021	0.020

Treatment	Mn		Fe		Cu		Zn		B		Al	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
	ppm		*ppm*		*ppm*		*ppm*		*ppm*		*ppm*	
Low N	45.6	214	64.6	1720	14.9	94.5	75.3	422	163.4	24.8	143	586
Intermediate N	42.6	249	65.4	1740	12.3	160.4	59.2	434	153.3	25.7	119	562
High N	42.2	308	69.4	1890	12.9	218.2	45.7	565	121.6	25.4	104	653
Low K	41.7	256	67.0	1730	13.0	152.1	58.3	462	163.3	25.1	116	575
Intermediate K	44.4	243	65.2	1700	13.3	149.1	59.9	479	148.2	25.2	123	590
High K	44.3	271	67.2	1920	13.8	171.9	62.1	479	126.8	25.7	127	636
Low Mg	40.9	263	67.6	1650	14.8	154.5	56.5	546	147.9	25.2	130	616
Intermediate Mg	41.5	253	68.8	1860	12.9	167.2	57.7	449	149.4	25.0	123	576
High Mg	48.0	254	63.1	1840	12.4	151.4	66.0	426	141.0	25.7	113	609
L.S.D. @ 5%	3.2	17.2	N.S.	N.S.	1.4	24.8	6.1	54	9.9	N.S.	12.2	72
L.S.D. @ 1%	4.2	25.2	N.S.	N.S.	1.8	32.8	8.0	72	13.1	N.S.	16.1	95

* Micro-elements in leaves are from leaf samples taken in July, 1948; all other determinations are from final harvest in January, 1951.

YSES: The main-effect means of the macro-nutrient elements determined on the leaves and small roots at final harvest are shown in table II. The micro-element values for leaves are from a previous sampling in which the leaves were washed. Each value presented is a mean of 36 determinations, the large number resulting from the internal replications in this type of experiment. The leaf values are rather typical of determinations made in the various samplings during the course of the experiment. While mean values mask the extreme values of individual treatments, they illustrate the main features.

The data are largely self-explanatory. It will be noted that the fibrous root values for macro-elements are usually smaller than the leaf values and often they reflect the same trend. There are, however, important exceptions that may have fundamental implications. For example, increased K depressed Ca in the leaf but had no such effect on Ca in the root. Conversely, K level had relatively small influences on leaf Mg and Na but greatly affected the rootlet concentrations of these elements. Similarly, the effect of N supply on leaf Ca was large, but N had only a slight effect on root Ca. Both Mg and Na showed opposite trends in the leaves and roots in relation to N level. The Mg concentration in the roots showed a much narrower range in relation to Mg supply than it did in the leaves. As the supply of Mg was increased leaf K declined substantially, but root K was virtually unchanged.

These results indicate the complexity of ion accumulation in various plant parts. The Ca-K-Mg relations in leaves and roots show that entirely different ratios of these elements can be found in different parts of the same plants. This suggests that reciprocal cation substitution on a purely chemical basis may take place in all tissues as both roots and leaves of these citrus trees had nearly constant total equivalent cationic composition as shown later (tables IV and VI).

Several types of investigation show that there is a close relation between adsorption and movement of cations in plants and electrical potential gradients (2, 7, 9) and at least part of the adsorption activity of roots is not dissimilar to that of non-living exchange systems (13).

Two other tree fractions also were analyzed for the elements N, P, K, Ca, and Mg. Complete data are not presented for these, but they can be briefly summarized since the trunk, branch, and twig fraction (including both bark and wood) showed almost identical trends with those found for leaves, the chief difference being that all elements were present in lower concentrations. Likewise the large roots showed the same trends as those shown for the small fibrous roots but the concentrations were lower. All 4 tree fractions thus showed inverse N-P and N-K concentrations. Above-ground parts showed a strong reciprocation Ca-K concentrations and weak reciprocation between K and Mg whereas the underground parts showed no depression of Ca by K but a strong reciprocation between K and Mg.

The heavy-metal elements were all found to be present in several-fold concentration in the roots as compared with leaves. Nitrogen level in particular had a strong influence on these elements. It was previously shown that a parallel exists between protein content of roots and Cu accumulation (15). Evidently this same relation is true for Zn and Mn. It is perhaps noteworthy that the root concentration of each of these heavy metals seems to have no direct relation to the leaf concentration. These elements are all readily adsorbed by both living and dead roots (15). Copper, at least, is held in such an exchangeable manner that it can be displaced readily by dilute acid (15), but much less readily by neutral base elements.

The depressive effect of N on the leaf concentrations of most of the micro-elements is not readily explained. It may be related to the greater growth and fruit production associated with increased N. If so, such an explanation should apply also to Fe but it doesn't. Likewise, some of the effects of K and Mg on the concentrations of the micro-elements are not readily understood. For example, increased K caused a depression in leaf B and increased Mg caused an increase in leaf concentrations of Zn and Mn while depressing Cu. The effect of Mg on Zn and Mn has shown up visibly in Florida orchards in conjunction with the overcoming of Mg deficiency (4). The explanation offered was that more vigorous root systems developed when Mg was supplied to deficient trees and this enhanced their foraging ability for Zn and Mn. Under the present conditions Mg was not found to stimulate root growth (18), but it still increased the movement of these elements into the leaves.

These comparative data show that an evaluation of either leaf or root composition, alone, may lead to different conclusions. It is possible that for single element studies with N, P, or K almost any part of the plant would serve as a satisfactory indication of nutritional level. This has been shown with citrus in the case of K (5). However, for total base relations or micro-element effects it would seem desirable to consider also rootlet composition rather than to rely wholly on foliar composition in evaluating the nutritional status of plants.

INTERACTIONS OF VARIABLE ELEMENTS ON TISSUE COMPOSITION: One of the primary reasons for using a factorial design in experiments is to detect and measure the interactions of various treatments. For instance, it may be desired to see whether nitrogen rate has the same effect under conditions of high K as it does under those of low K. As reported previously (18), there were very few interactions in regard to tree growth, fruiting, or fruit quality among the three elements under study. In contrast, mineral analyses of the leaves and fibrous roots show numerous interactions. Some of the more definite ones are listed in table III. Those listed for leaf analyses are usually the ones that consistently appeared in several samplings. The root interactions are of course from only the samples taken at final harvest. The

TABLE III

INTERACTIONS OF VARIABLE ELEMENTS ON THE CONCENTRATIONS OF ELEMENTS FOUND IN LEAVES AND FIBROUS ROOTS

PLANT PART AND ELEMENT IN ANALYSIS	INTERACTING ELEMENTS IN TREATMENT	DESCRIPTION OF INTERACTION
		Leaves
K	N K†	N depressed K at low K; not at high K
K	N Mg**	Mg depressed K at low N; not at high N
P	N Mg**	Mg depressed P at low N; not at high N
Mg	N K**	K depressed Mg at intermediate and high N; not at low N
Mg	N Mg**	N increased Mg at high N; not at low or intermediate N
Ca	N K**	N increased Ca at low and intermediate K; not at high K
Ca	K Mg**	Mg depressed Ca at low K; increased Ca at high K
Na	N K†	K depressed Na more at intermediate N than at low or high N
Mn	K Mg*	Mg increased Mn more at low K than at high K
		Fibrous roots
K	N K†	N depressed K at low and intermediate K; not at high K
Mg	N K*	K depressed Mg more at low N than at high N
Mg	N Mg**	N increased Mg more at low Mg than at intermediate or high Mg
Ca	N Mg*	Mg depressed Ca at low N; not at high N
Na	N K†	K depressed Na more at intermediate N than at low or high N
Na	K Mg*	Mg depressed Na more at intermediate and high K than at low K
Cu	N K**	K depressed Cu at high N; increased Cu at low N
Cu	N Mg*	Mg depressed Cu at high N; increased Cu at low N
Mn	N K**	K depressed Mn at high N; increased Mn at low and intermediate N
Mn	N Mg**	Mg depressed Mn at high N; increased Mn at low and intermediate N
Zn	N Mg†	Mg depressed Zn at high N; not at low or intermediate N
B	N K†	N depressed B at low K; increased B at high K
B	N Mg**	N depressed B at low Mg; increased B at high Mg
Al	N K*	K depressed Al at low N; increased Al at intermediate and high N

* Indicates significance at 5%.
** Indicates significance at 1%.
† Indicates significance at 0.1%.

first interaction, for illustration, indicates that increased N depressed the concentration of K in the leaves when K was supplied at a low level but not when it was at a high level. The N-K interaction on Na in leaves is peculiar in that K showed a much stronger depressive action against Na at the intermediate N level than at either the low or the high N levels. The reason for this is not apparent. Few of the interactions can be logically explained in the light of present knowledge. Apparently chemical reactions and vital functions are interwoven in a complex pattern. Some interactions doubtlessly are of significance in understanding plant behavior; others probably are incidental and may occur only under a particular set of environmental conditions.

Regardless of the cause of interactions, it is of importance that they be recognized in interpretation of experimental results. For example, if two experiments were conducted with different levels of Mg, one at a very low K level and another at a high, opposite conclusions probably would be reached concerning the effect of Mg on the Ca content of leaves. This can be noted by observing the K-Mg interaction for leaves in table III, which indicates a depression of Ca by Mg when K supply is low but an accentuation when K supply is high. This clearly illustrates how an experiment not designed to evaluate possible interactions can give results that may be misleading.

RECIPROCATION OF BASE ELEMENTS: Studies of a number of agronomic plants show that an interrelation exists among the elements K, Ca, and Mg in tissues. Sodium may also enter the picture if it reaches the foliage in appreciable quantities. Examples and discussion of base reciprocation with a review of pertinent literature were given by Lucas and Scarseth (11). Little attention has been given to total base content and base substitution in tree crops. Numerous workers, however, have shown that high K usually depresses Ca and vice versa without regard to total base equivalence. Shear et al. (14) pointed out that total cation equivalents may not be constant owing to fluctuation in total anions. Cain (3) found that the leaf cations of apples may show a nearly constant total equivalent value, but the woody shoots showed less tendency for base constancy. Fudge (8) noted that when K fertilization of citrus was increased both Ca and Mg were reduced in the leaves. The shifts in concentration suggest chemical equivalence although Fudge's data showed there was a tendency for the total base equivalents to increase as the Ca concentration in the leaf increased.

The data in table IV are from analyses made on the entire leaf population at final harvest. Each determination is a mean of the 4 replicates for a given treatment. The data are arranged in ascending order on the basis of the Ca content. The treatments are not listed since leaves produced in all treatments tended to show that the total of base equivalents was constant except when Ca was high as described subsequently. The main reciprocation shown is between Ca and K although Mg is somewhat of a factor, particularly when the K concentration is low. These data, as well as those for other sampling dates, seem to indicate a base

constancy of about 200 mEq per 100 gm of dry leaf tissue when Ca is below 3.5 % (or about 170 mEq per 100 gm). With greater Ca concentrations the total milliequivalents increased as though some insoluble Ca salt was being precipitated. Calcium oxalate crystals are known to exist in citrus leaf cells (13). A similar relation was noted in spinach (1) in which the total cation concentration increased at high Ca levels. Calcium in citrus leaves from Florida orchards is frequently found to be present in amounts of 3 to 4 %, but is usually much higher in other citrus-growing areas of the United States. The total base composition in these latter areas is not a constant but continues to increase in proportion to the Ca content. It is felt, however, that the data in table IV substantiate the opinion of others (3, 10) that the several base cations may interchangeably serve certain functions in a leaf.

Consideration of leaf analysis alone may give the wrong impression of reciprocation in other plant parts. Data from fibrous roots also showed essentially constant totals of base equivalents; however, the reciprocation found was entirely between K and Mg (table V). The total mEq per 100 gm of dry roots was about 155 as compared with about 200 or more in the leaves. Smaller constant values are seen for above-ground and below-ground woody structures of the trees. These data show that constant base equivalents exist for all parts of the tree when it is grown under sand culture conditions with widely different K levels. Similar total mEq values are found in relation to the 3 Mg levels but different reciprocations are emphasized. The data are not presented, as only two important differences appeared: (a) in all tree fractions except the leaves, K was constant and the reciprocation was between Mg and Ca; (b) in the leaves a doubling of Mg concentration resulted in almost equal depression of Ca and K.

In contrast to these base relations in trees of equal size, a somewhat different picture is formed when the base values for trees grown on different N levels are compared (table VI). Nitrogen greatly affected growth, the high-N trees being more than twice as large as the low (18). The total bases per unit weight in leaves increase with N supply but may be considered constant if allowance is made for Ca (presumably precipitated) in excess of about 170 mEq as shown in table IV. The base totals in the fibrous roots are irregular. The woody structures, however, show a steady decrease in total bases as though the greater growth caused a dilution of the bases. These data suggest, therefore, that base equivalents in leaves might be compared in trees with widely different N statuses but such comparisons between other tree parts might be expected to show differences in total bases per unit weight depending on tree vigor, amount of growth and other factors associated with N response.

Leaf analysis has provided a very useful research tool. It has generally been assumed that tissue analysis, as an indicator of nutritional status of a plant, is at least empirically related to the mineral composition of the rest of the plant. The information

TABLE IV

RECIPROCAL RELATION OF BASE ELEMENTS IN LEAVES OF TREES GROWN IN SAND CULTURE WITH DIFFERENTIAL NITROGEN, POTASSIUM, AND MAGNESIUM

% IN LEAVES			MEQ PER 100 GM			
Ca	K	Mg	Ca	K	Mg	Total
2.1	2.6	0.28	105	66	23	194
2.2	2.5	0.37	110	64	30	204
2.4	2.0	0.18	120	51	15	186
2.6	1.95	0.26	130	50	21	201
2.7	1.45	0.40	135	37	33	205
2.8	1.2	0.40	140	31	33	201
3.0	1.2	0.20	150	31	16	198
3.2	1.0	0.20	160	26	16	202
3.4	0.7	0.25	170	18	21	209
3.8	0.6	0.25	190	15	21	226
4.0	0.4	0.50	200	10	41	251
4.5	0.4	0.30	225	10	25	260
4.8	0.45	0.15	240	11	12	263

TABLE V

BASE COMPOSITION (MEQ PER 100 GM DRY TISSUE) OF VARIOUS CITRUS TREE FRACTIONS AS AFFECTED BY POTASSIUM LEVEL

Treatment	Leaves				Fibrous roots			
	Ca	K	Mg	Total	Ca	K	Mg	Total
Low K ..	185	21	25	231	100	15	39	154
Inter. K ..	171	28	23	222	100	21	33	154
High K ..	130	46	21	197	100	39	16	155
	Topwood				Rootwood			
	Ca	K	Mg	Total	Ca	K	Mg	Total
Low K ..	55	6	11	73	36	8	6	50
Inter. K ..	56	8	10	74	36	10	6	52
High K ..	48	13	9	70	34	13	4	51

TABLE VI

BASE COMPOSITION (MEQ PER 100 GM DRY TISSUE) OF VARIOUS CITRUS TREE FRACTIONS AS AFFECTED BY NITROGEN LEVEL

Treatment	Leaves				Fibrous roots			
	Ca	K	Mg	Total	Ca	K	Mg	Total
Low N ..	127	51	24	202	92	29	24	145
Inter. N ..	162	28	22	212	95	26	33	174
High N ..	197	15	22	234	110	21	31	162
	Topwood				Rootwood			
	Ca	K	Mg	Total	Ca	K	Mg	Total
Low N ..	65	14	10	89	37	14	6	57
Inter. N ..	52	8	10	70	35	11	6	52
High N ..	42	5	10	57	32	7	5	44

reported here, however, indicates some of the limitations to interpretations of leaf analysis data. Base reciprocation in leaves may be of an entirely different character, both quantitatively and qualitatively, from that in other parts of the plant. Leaf content of B and the metallic elements may bear no apparent relation to the concentrations of these elements in another tissue, such as the fine roots. This situation may make it desirable to consider the distribution of elements in various parts of the plant before conclusions are drawn in regard to nutrient availability, absorption behavior, and the location of antagonistic reactions. A practical example of the failure of leaf analysis has been experienced in Florida in recent years in connection with acid-soil chlorosis of citrus. Leaf analysis indicated that Fe was deficient but that Cu was present in normal amounts (19). Further investigations (12, 17) have shown that the primary cause of the trouble is Cu toxicity to the roots, a consequence of rather large accumulations of applied Cu.

Summary

Chemical analyses were made on leaves, trunk and branches, large roots, and fibrous roots of budded Valencia orange trees grown in sand culture with different levels of N, K, and Mg.

Leaf samples taken 3.5 months after the start of treatments showed that the ranges of N and K concentrations were about the same as those shown by subsequent samplings for later years. Leaf Mg, however, showed little change after this length of time but was found to be in equilibrium with the rate of supply after about one year.

Comparisons of the concentrations of 12 mineral elements in leaves and fibrous roots showed quantitative differences in all. Nitrogen, P, and K values in the two tissues appeared to parallel one another fairly closely although the roots contained lower levels of these elements. Analyses for the other 9 elements showed important discrepancies, either in amounts or relation to treatment, between these two tissues.

Numerous interactions were found between N and either K or Mg on the concentrations of various elements in both roots and leaves. This indicates the desirability of studying N in factorial experiments, as its effects on tissue composition may vary appreciably with different supplies of other elements. Potassium and Mg, on the other hand, showed very few interactions, indicating that single variable studies involving these elements would usually give reliable information.

Base reciprocation was evident in all parts of the tree. The nature and extent of these varied from one part of the tree to another and they were influenced by differences in supply rates of all three variable elements. Under the conditions of this study the leaves tended to show a constant total equivalent content of Ca, K, and Mg except when calcium exceeded about 170 mEq per 100 gm dry weight regardless of treatment. Constant equivalent totals for other tree parts occurred at all levels of K and Mg, where the amount of tree growth was nearly equal, but did not in relation to nitrogen level which greatly affected growth. Greater growth of the tree tended to dilute the base elements in tissues other than the leaves.

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THE INFLUENCE OF MINERAL NUTRITION ON THE ORGANIC ACIDS OF THE TOMATO, LYCOPERSICUM ESCULENTUM[1,2]

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Making use of tomato fruits (and to lesser extent other plant parts) which were grown primarily for another project[3] in the Department of Horticulture, it was proposed to take advantage of recent developments in the determination of individual organic acids (1, 2) to determine, what, if any, effects different fertilizer treatments might have on the quantities and kinds of organic acids present. No absolute deficiencies were sought. Rather it was desired to find out whether certain variations in the quantities of several common elements, as might occur in horticultural practice, could appreciably influence the composition of a crop plant such as the tomato; and more particularly, if differences appeared, whether these were primarily associated with the effect of one element or of various combinations of different elements.

Furthermore, until recently, despite the great volume of published work, there has been little reliable information about the occurrence of individual organic acids in plants (3, 4, 5, 6).

As to the roles of minerals in plant metabolism, much has been learned in recent years, particularly the significance of phosphorus compounds in respiration and photosynthesis; but there is little information about the role of potassium (7); and very little has been found out about the intermediate metabolism of nitrogen (8). Pepkowitz et al (9) in a study of the importance of oxygen in culture substrates for oats, reported that the nitrate level as well as the oxygen level affected the nature and amounts of organic acids present. Rapid absorption, translocation, and assimilation of the ammonium ion with considerable amide production was noted by MacVicar and Burris (10). Tiedjens (11) showed that the pH of the medium affects the assimilation of nitrate and ammonium nitrogen by the tomato. Clark (12) found that tomatoes grown in sand culture and supplied with ammonium ion may contain less than one tenth as much of each individual organic acid as do plants supplied with nitrate nitrogen. Wadleigh and Shive (13) also observed that the form of nitrogen supplied (as well as the pH) influenced the organic acid content of corn. Wall (7) found that tomatoes deficient in potassium usually contain a higher percentage of soluble nitrogenous compounds than plants supplied with adequate potassium and this has been found to hold true for many other species of plants. With beets, Lyubarskaya (14) reports that KCl seemed to increase the uptake of nitrates and ammonia. Lee and Sayre (15) and Culvern (16) found a higher total acidity results from increased potassium fertilization of tomatoes. Breon and Gillam (17) studied the influence of phosphorous and the form of available nitrogen on the nitrogen metabolism of the tomato. Higher concentration of soluble nitrogen compounds always accompanies phosphorus starvation (18) while increased acidity results (19).

No complete analysis of amino acid composition of the tomato, plant or fruit, could be found in the literature, although there are many reports of the presence of individual acids, especially glutamic acid and tryptophane (10). Vickery (20) found a large amount of glutamine when ammonium sulfate was used, but comparatively little in calcium fertilized tomato plants.

Experimental

Materials for this study were supplied by the Department of Horticulture greenhouse at The Ohio State University. The variety of tomato was wilt-resistant Globe. The plants were grown in raised "Haydite" beds. The latter is a burned shale product widely used in greenhouses instead of gravel for culture work of this kind. These beds were irrigated with culture solutions of known composition once or twice daily. The amounts of the various elements in the solutions were checked once every two weeks and suitable adjustments were made to maintain uniform concentrations. In general the compositions of the different solutions were the same, except for the variations in the level of one or more of the elements, P, K or N. All the elements known to be essential to plant growth were supplied and the pH was kept around 6.5 by the addition of suitable quantities of

[1] Received November 9, 1953.

[2] This work was partly supported by a grant from the Ohio State Research Foundation funds for aid in fundamental research.

[3] Project No. 234, Ohio Agricultural Experiment Station.

HCl or NaOH. Thus in a typical experiment, the phosphate, Ca and Mg levels were kept constant at 100, 200, and 25 ppm, respectively, and the N and K levels varied, as 50, 200; 50, 400; 100, 200; or 100, 400 ppm N and K in the individual beds. Nitrate was supplied in the form of calcium nitrate and any discrepancy in Ca level was made up by the addition of calcium sulfate. With K, the required amount of phosphate was first satisfied in the form of mono potassium phosphate; KCl was then added to yield the required K level. Trace elements, Mn, B, Cu, and Zn, were included in each solution at concentrations of 0.5, 1.0, 0.5 and 0.5 ppm in the form of appropriate compounds; iron salts were added each week to keep a concentration of 2 ppm of iron. The concentrations of only the elements that were varied are given in the legends of the figures.

With ripe tomatoes, quarters from several fruits were used, the wall and pulp separated with a spatula and each fraction pressed through cheesecloth to exclude the skin and seeds. The weighed juice samples were placed directly in the containers which fitted to the ground glass joints of the freeze-drying apparatus. To facilitate drying, this material was evenly spread over the inner surface by rotating over dry ice. After lyophilizing for 6 to 7 hours, the samples were found to be sticking to the sides of the containers and to be very hygroscopic. They were therefore immediately acidified with sulfuric acid (about 0.5 ml of 4 *N*), mixing thoroughly with a glass rod. Addition of 1 to 2 gm of silica gel aided in the transfer of these acidified samples to the extraction thimbles and also adsorbed any water. The thimbles were placed in a desiccator until ready for extraction. Root and leaf samples were ground in a mortar surrounded by dry ice. The weighed samples were placed in the extractor thimbles directly and lyophilized.

Alcohol-free ether (permanganate washed) was used for the extraction, which was found to be complete after 6 to 8 hours. The ether layer was thoroughly extracted with slightly alkaline distilled water until it was completely freed from any traces of acids. These aqueous extracts were then combined and evaporated in a small beaker placed in a stream of air on a hot plate at low heat. The resulting dry sodium salts were stored in a desiccator until needed.

When desired, the above samples were made up to a definite volume (as 25 ml) with distilled water. Suitable aliquots of these sodium salts of the acids (which should contain 10 to 100 mg of free organic acids after liberation from their salts) were pipetted into small beakers and dried as above described. To the dry salts were then added 0.5 ml of 4 *N* sulfuric acid and 1 gm of dry silica gel. This mixture was then transferred to the top of the silica gel column for chromatography. In the first part of this study, citric acid was determined separately because of the capacity of the column used and its relative abundance in the samples, compared with the other acids. Later it was eluted first from a shorter column (12.5 cm).

The chromatographic method, using a silica gel column with butanol-chloroform mixtures for developing agents as described by Bulen et al (1), was then employed for the determination of the nitrogen-free organic acids. Fractions were collected through the use of the special fraction collector devised by Varner and Bulen (21).

The separation of amino acids was effected by elution with various buffers on long and short Dowex-50 columns, following the procedure of Moore and Stein (2). The concentration was determined by use of the photometric ninhydrin method (22). Before determinations were made with tomato samples, all the columns used were calibrated with known amino acid mixtures.

TABLE I

EFFECTS OF NITRATE AND POTASSIUM ON THE ORGANIC ACIDS OF RIPE TOMATO FRUITS (SPRING CROP—1951)

Fertilizer treatment			Fruit part	Organic acids						
NO_3^-	K^+			Acetic	Formic	Lactic (?)	Trans-aconitic	Malic	Citric	Total
ppm				*mEq × 10³/10 gm fresh weight*						
50	200	A	Pulp	1.5	2.4	1.1	0.6	4.1	157.1	166.8
			Wall	1.8	2.9	0.8	3.0	4.0	63.9	76.4
50	400	B	Pulp	1.6	3.3	0.6	0.4	3.5	166.0	175.4
			Wall	2.2	2.6	1.5	1.9	4.0	71.3	83.5
100	200	C	Pulp	2.3	3.4	1.6	0.7	3.7	166.2	177.9
			Wall	3.2	3.0	1.1	1.8	3.7	70.5	82.8
100	400	D	Pulp	2.8	2.8	1.0	2.5	4.4	166.8	181.3
			Wall	4.4	2.1	1.3	2.9	3.5	79.1	84.8
200	200	E	Pulp	2.6	4.1	3.6	1.4	2.7	178.3	193.7
			Wall	3.2	2.2	1.3	4.9	5.1	106.3	124.0
200	400	F	Pulp	2.8	4.0	1.3	2.7	5.6	214.4	230.8
			Wall	2.5	3.4	1.2	8.2	7.5	134.0	156.8

Some determinations were made on fruits which had been infiltrated with, or injected with, known salt solutions and these fruits then allowed to ripen for certain periods of time before analysis.

Some experiments with vacuum infiltration were finally rejected because of Gustafson's report (23) that with vacuum infiltration, anaerobic respiration is greatly increased over the usual amount of aerobic respiration.

So many data were accumulated that it was thought best to present the results in the form of charts. Figure 1 represents table I transformed to chart form. It shows the effects of varying levels of nitrate and K on the organic acids of ripe tomato fruits. The other figures were made in the same way and each is provided with an appropriate description.

Discussion of Results

The same organic acids found by Bulen (6), viz., formic, acetic, lactic (identification in doubt because of very small amount), trans-aconitic, malic, and citric, were found in the ripe fruits of the wilt-resistant Globe tomato. In some of the separations, an acid coming off after trans-aconitic was noted which was present in very small quantities and was only found in 10 out of 32 chromatographic separations. This acid could be glycolic, cis-aconitic, or oxalic.

Based on the analysis of separate samples of the gelatinous pulp and the fruit wall of vine ripened tomatoes, the gelatinous pulp has more total organic acids than the fruit wall. In interpreting these results, however, it should be noted that in both instances a part of the acids reported is present in the fruit in the form of salts rather than as free acids. Citric acid is the predominant acid in ripe tomatoes. The gelatinous pulp contained as much as 1.5 to 2.5 times as much citric acid as the fruit wall, depending upon fertilizer treatment.

Although higher levels of nitrate (fig 1-E and -F, 2-E) give rise to greater amounts of citric and total N-free acids than when the N is supplied as ammonium ion (fig 2-A and -B), nevertheless other ions as K^+ and phosphate greatly influence the acid production, especially at a low nitrate level. Thus (fig 3-D and -E) increase of K seems to be just as effective as increase of nitrate (fig 3-C) in raising the organic acid content. The organic acid content does not seem to be primarily influenced by a single element; nor is it always an effect of the form of combined N supplied. There rather appear to be intereffects and similar effects of entirely different combinations of the ions required for growth. Although ammonium N (fig 2-A and -B) gives rise to smaller amounts of citric and of total organic acids than does 200 ppm nitrate (fig 2-E), the lower nitrate levels, 50 and 100 ppm (fig 2-C and -D) show less of these acids than do the ammonium ion supplied plants. Acetic acid appears to be relatively higher in the whole fruit of the ammonium ion treated plants (fig 2-A and -B). Also, examining figure 1-F, where both K and nitrate are relatively high compared with the other treatments, it will be noted that an increase is evident for most of the N-free acids. Thus K and nitrate may mutually supplement the effect of each other.

The above results are all for ripe fruits. A few results are given for green fruits in figure 4. Here, increased nitrate (fig 4-C) gives rise to a high trans-aconitic acid content, but the other acids do not vary greatly with the specified treatments. At all nitrate levels used, the roots and leaves are characterized by larger quantities of trans-aconitic and acetic acids than the fruits. With the latter, malic and citric acids seem to be the quantitatively important acids.

Using 2.5 gm of pressed juice as samples, the following free amino acids were found present: glu-

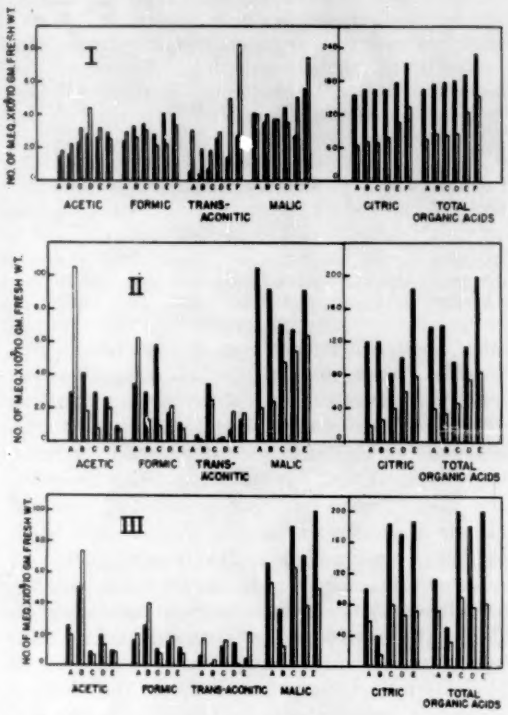

Fig. 1. Effects of nitrate and K levels on the organic acids of ripe tomato fruits (spring crop, 1951). Ppm N and K respectively: A, 50–200; B, 50–400; C, 100–200; D, 100–400; E, 200–200; F, 200–400. Closed bar, gelatinous pulp; open bar, fleshy wall.

Fig. 2. Effects of nitrate and ammonium N levels on the organic acids of ripe tomato fruits (fall crop, 1952). A, 50; B, 100 ppm ammonium N; C, 50; D, 100; and E, 200 ppm nitrate N. Closed bar, pulp; open bar, wall.

Fig. 3. Effects of K and P levels on the organic acids of ripe tomato fruits (fall crop, 1951). Ppm N (supplied as nitrate), K, and P respectively: A, 100–200–100; B, 100–200–200; C, 200–200–100; D, 100–400–100; E, 100–400–200. Closed bar, pulp; open bar, wall.

TABLE II

EFFECTS OF AMMONIUM AND NITRATE NITROGEN, POTASSIUM AND PHOSPHORUS ON THE FREE AMINO ACIDS OF THE FRUIT PULP OF RIPE TOMATO FRUITS (FALL CROP—1951)

AMINO ACID	FERTILIZER TREATMENT							
	NH_4^+— 50 K —200 P —100	NH_4^+—100 K —200 P —100	NO_3^-— 50 K —200 P —100	NO_3^-—100 K —200 P —100	NO_3^-—200 K —200 P —100	NO_3^-—100 K —400 P —200	NO_3^-—100 K —400 P —100	NO_3^-—100 K —200 P —100
	mg/10 gm fresh weight							
Aspartic	2.16	0.72	0.77	1.92	2.08	1.68	2.08	2.56
Serine	0.94	0.40	0.96	0.94	2.83	1.36	1.24	1.40
Glutamic	10.12	0.96	10.40	12.80	16.56	13.98	14.59	15.47
Alanine	0.57	0.32	0.30	0.77	0.72	0.42	0.42	0.61
Valine	0.49	0.54	Trace	Trace	Trace	0.55	Trace	0.66
Leucine	Trace	Trace	0.60	0.43	Trace	0.87	0.50	0.85
Phenylalanine and Tyrosine	1.82	Trace	Trace	1.46	Trace	1.31	1.41	1.21
Tryptophane	8.00	0.74	0.48	3.23	4.92	5.96	0.64	4.28
Histidine	0.80	1.98	1.04	0.60	0.52	7.48	0.65	1.36
Lysine	0.32	0.51	0.50	0.55	1.17	0.38	1.82	0.83
Arginine	0.30	0.97	0.92	0.52	0.38	1.02	1.12	0.70

tamic, aspartic, tryptophane, phenylalanine (plus tyrosine), serine, histidine, valine, alanine, leucine, lysine, and arginine. Some of the samples appeared to contain traces of methionine, cystine, glycine, and isoleucine. A ninhydrin-reacting substance, emerging before aspartic acid, was also found which was probably urea. Proline could not be definitely identified because of the large amount of glutamic acid which overlapped with proline in the chromatogram. As was true with anazotic acids, the gelatinous pulp has more free amino acids than the fruit wall (tables II, III). The predominantly occurring amino acids were glutamic, aspartic, tryptophane, serine, phenylalanine (plus tyrosine), and histidine. Doubling the ammonium ion concentration appeared to have a pronounced effect on the amount of tryptophane and glutamic acid formed.

It is interesting to note that pyruvic and α-ketoglutaric acids, known participants in the Krebs respiration cycle, were not identified and if present must have occurred in extremely small quantities.

As the tomato fruit develops, the content of organic acids (and their salts) increases and then finally decreases as the ripening proceeds (fig 5). This is in agreement with several other reports in the literature. From the data here given it will be noted that the immature fruits have a higher volatile acid and a lower citric acid content than do the matured but still unripe fruits. Citric acid decreases in the fully ripe fruits.

Mature green fruits injected with various N, P, and K salts and allowed to ripen artificially showed, in general, no definite correlation with any particular acid. Certain ammonium ion-containing solutions

TABLE III

EFFECTS OF AMMONIUM AND NITRATE NITROGEN, POTASSIUM AND PHOSPHORUS ON THE FREE AMINO ACIDS OF THE FRUIT WALL OF RIPE TOMATO FRUITS (FALL CROP—1951)

AMINO ACID	FERTILIZER TREATMENT							
	NH_4^+— 50 K —200 P —100	NH_4^+—100 K —200 P —100	NO_3^-— 50 K —200 P —100	NO_3^-—100 K —200 P —100	NO_3^-—200 K —200 P —100	NO_3^-—100 K —400 P —200	NO_3^-—100 K —400 P —100	NO_3^-—100 K —200 P —100
	mg/10 gm fresh weight							
Aspartic	1.52	0.88	1.23	2.56	0.23	1.76	0.88	3.20
Serine	1.77	0.73	0.80	1.42	2.12	1.76	1.30	1.12
Glutamic	8.33	0.87	2.83	8.32	12.48	10.71	11.31	9.60
Alanine	0.58	0.30	0.33	0.30	0.28	0.37	0.72	0.42
Valine	0.73	Trace	0.67	Trace	Trace	0.60	Trace	Trace
Leucine	0.82	0.94	0.50	Trace	0.57	0.93	Trace	0.60
Phenylalanine and Tyrosine	1.82	0.99	1.07	1.17	1.38	1.51	0.64	1.64
Tryptophane	5.62	0.99	0.12	0.87	2.57	0.68	4.42	2.17
Histidine	0.96	0.33	0.88	2.03	1.13	4.44	0.64	0.94
Lysine	0.84	0.16	0.64	1.04	0.99	0.68	1.61	1.04
Arginine	0.72	0.78	0.44	Trace	Trace	0.89	0.90	Trace

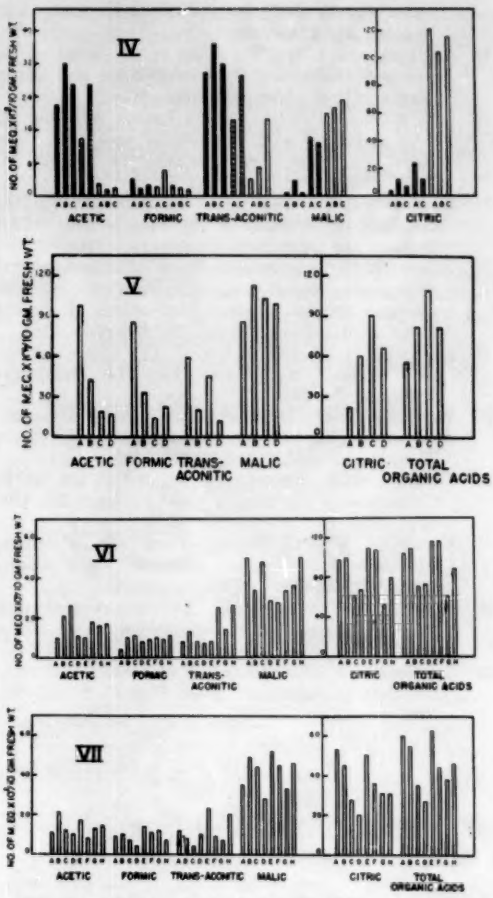

FIG. 4. Effects of nitrate levels on the organic acids of the roots, leaves and green fruits of the tomato. Ppm nitrate N: A, 50; B, 100; C, 200. Closed bar, roots; cross hatched bar, leaves; open bar, fruits.

FIG. 5. Changes in the organic acids during the development and ripening of tomato fruits. A, small unripe; B, C, not fully ripe; D, fully ripe.

FIG. 6. Effects of directly infiltrated nitrogenous salts on the organic acids of the gelatinous pulp of ripened tomato fruits. A, control; B, water; C, $NaNO_3$; D, KNO_3; E, $Ca(NO_3)_2$; F, NH_4NO_3; G, $NH_4NO_3 + (NH_4)_2SO_4$; H, $(NH_4)_2SO_4$.

FIG. 7. Effects of directly infiltrated nitrogenous salts on the organic acids of the fleshy wall of ripened tomato fruits. A, control; B, water; C, $NaNO_3$; D, KNO_3; E, $Ca(NO_3)_2$; F, NH_4NO_3; G, $NH_4NO_3 + (NH_4)_2SO_4$; H, $(NH_4)_2SO_4$.

did give lower citric and total acid values (figs 6, 7-G and -H) than various other treatments including the control; but sodium and potassium nitrate infiltrated fruits (fig 6, 7-C and -D) also showed low citric and total acid values.

SUMMARY

1. Formic, acetic, trans-aconitic, malic, citric, and possibly cis-aconitic acids occur in the roots, leaves, and fruits of the wilt-resistant Globe tomato.

2. There are more acetic and trans-aconitic acids and less malic and citric acids in the roots than in the fruits, with intermediate amounts in the leaves.

3. The gelatinous pulp of vine-ripened tomato fruits contains larger amounts of acids than the fruit wall. Specifically there are more citric, acetic, formic and malic acids in the gelatinous pulp than in the fruit wall of ripe tomatoes. These acids, of course, in all instances mentioned, are partly in the form of their salts in the original fruit samples.

4. The effects of various ions on the concentrations of anazotic acids in tomato fruits does not seem to be a specific effect of certain particular ions as nitrate or ammonium, but rather an interrelated effect. It is also dependent upon concentrations of these ions. Therefore, statements such as "nitrate always favors acid production, while the ammonium ion has the reverse effect," cannot be justified.

5. The predominantly occurring amino acids of the ripe tomato fruit are: glutamic, aspartic, tryptophane, phenylalanine (plus tyrosine), and histidine. The gelatinous pulp has a higher free amino acid content than the fruit wall. Doubling the concentration of ammonium ion appears to have a marked effect on the amounts of tryptophane and glutamic acid.

6. Changes in the acids present occur during the development and ripening of tomato fruits. Acetic acid decreases while citric acid increases up to some stage in the ripening when the latter starts to decrease.

7. It was not possible to establish any very definite effect on the kinds or amounts of acids present in mature, ripening fruits which had been injected with various salt solutions.

8. Based on a summation of the various analyses of organic acids that were carried out it is believed that citric, acetic, and possibly malic acids are metabolically the most important anazotic acids in the tomato fruit. Especially when various plant parts are considered, a low citric acid content is frequently accompanied by a relatively higher acetic acid content.

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NITROGENOUS CONSTITUENTS OF SAP EXUDED FROM THE SAPWOOD OF ACER SACCHARUM[1,2]

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The large flow of sap which occurs under special conditions from the severed vessels of the sugar maple, *Acer saccharum*, and some other species of Acer, has been known for centuries. Work at the University of Vermont (6, 7, 8), which has been in progress for over fifty years, has dealt with many phases of this subject yet little attention has been given to the nitrogen compounds occurring in sap. The present investigation utilizes, apparently for the first time, recently developed techniques of paper chromatography to characterize some of the nitrogen compounds occurring in the sap. This represents a joint effort of the University of Vermont and Cornell University.[2]

The first reported figures for the nitrogen content of maple products were those of Hortvet (5) who found that protein ($N \times 6.25$) made up 0.223 to 0.334 % of maple syrup. It seems most unlikely that all the nitrogen as reported could have survived the prolonged boiling involved in syrup production in the form of protein. Jones and Bradlee (6) indicate a protein content of 0.0088 to 0.02 % in whole sap, again determined as $N \times 6.25$. These authors considered this amount quite low when compared with the protein content of wood of 0.71 to 1.38 % on a dry weight basis. Brodil (2) found one liter of sap to contain 0.19 % "protein substances" on a dry weight basis.

Recently Holgate (4) in a more detailed report gave nitrogen values for whole sterile sap as 0.003 to 0.008 %. He found that certain standard qualitative tests for both protein and nitrate were negative for the sap and for the scum which rises to the surface of the sap as it is being boiled down to maple syrup. Further, he found that the concentrating of sap to syrup also concentrates total N to an almost identi-

[1] Received January 14, 1954.

[2] The biochemical work in this paper was carried out at Cornell University under the general direction of Dr. F. C. Steward.

cal degree. Holgate concludes that the nitrogen was non-protein in character but was still organic.

Materials and Methods

All analyses were made on maple sap freshly collected in plastic sap buckets or one gallon glass jugs, quickly frozen in 300-ml aliquots and dried in a high vacuum blood plasma apparatus. By removing the loose bark on the tree with a draw-shave and painting the exposed surface with alcohol, drilling the tap hole with a sterile bit and collecting the sap in sterile containers, it was possible to maintain virtual asepsis. Plating out 10 ml aliquots on nutrient agar occasionally showed one or two bacterial colonies, but for analytical purposes the sap collected in this fashion was not influenced by the activity of microorganisms.

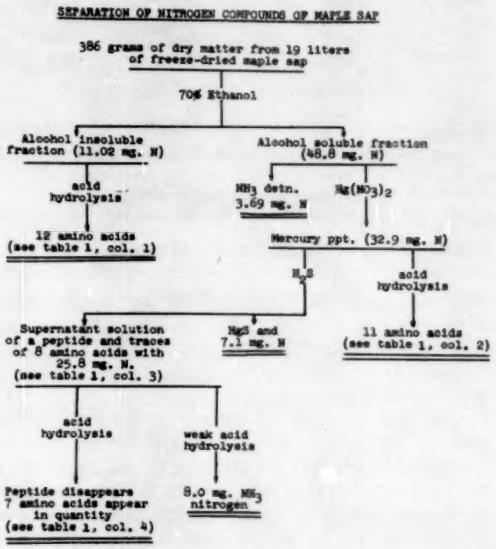
SEPARATION OF NITROGEN COMPOUNDS OF MAPLE SAP
386 grams of dry matter from 19 liters of freeze-dried maple sap
70% Ethanol
Alcohol insoluble fraction (11.02 mg. N)
Alcohol soluble fraction (48.8 mg. N)
acid hydrolysis
NH3 detn. 3.69 mg. N
Hg(NO3)2
12 amino acids (see table 1, col. 1)
Mercury ppt. (32.9 mg. N)
H2S
acid hydrolysis
Supernatant solution of a peptide and traces of 8 amino acids with 25.8 mg. N. (see table 1, col. 3)
HgS and 7.1 mg. N
11 amino acids (see table 1, col. 2)
acid hydrolysis
weak acid hydrolysis
Peptide disappears 7 amino acids appear in quantity (see table 1, col. 4)
8.0 mg. NH3 nitrogen

Fig. 1. Flow diagram for the fractionation of the nitrogen compounds of maple sap.

The analyses given were made after fractionating 386 gm of freeze-dried sap as shown in figure 1. This dry material represents about 19 l of whole sap. 70 % ethanol was used to fractionate the sap into alcohol soluble and alcohol insoluble portions. The alcohol insoluble material was centrifuged off, washed once with 70 % ethanol, dried, and then hydrolyzed with 6 *N* HCl overnight in an autoclave at 15 lb pressure. The free HCl was removed by repeated evaporation at room temperature in an air jet. The quantitative analyses for amino acids were done using the method of Thompson and Steward (14). Since qualitative tests for nitrate were negative, confirming the observation of Holgate (4), total N was determined by the standard Kjeldahl-Gunning method.

Partial precipitation of the soluble nitrogenous compounds was effected with mercuric nitrate reagent prepared according to the directions of Vickery (15). After washing, this precipitate was decomposed by suspension in 40 % ethanol and treatment with H_2S for 2 hours while stirring the suspension vigorously. HgS was centrifuged off, and the acidic solution neutralized. Aliquots were removed for determination of total N, amino acids, and easily hydrolyzed N (2 *N* H_2SO_4 for 2 hrs at 100° C). Another aliquot was removed for strong acid hydrolysis and subsequent chromatographic determination of the freed amino acids.

The ninhydrin method of Moore and Stein (10) for amino acids with unsubstituted amino groups and for ammonia was modified for use as a semi-quantitative assay for the sum of these groups. The modification consists of reacting a sample containing amino acids or ammonia with ninhydrin as described by Moore and Stein. The color developed is compared with ammonia standards, and the N so determined is then expressed as ammonia N.

A similar sample of maple sap concentrated by boiling was subjected to a partial analysis using some of the preceding methods.

Results

Alcohol Soluble Nitrogen: 48.8 mg of alcohol soluble N, representing 81.6 % of the N in maple sap, was sub-divided into three fractions as shown in figure 1. These fractions are, first, 25.8 mg of the soluble N precipitated by mercuric nitrate and resolubilized by treatment of the precipitate with H_2S, second, 7.1 mg of soluble N which is precipitated by mercuric nitrate but is not returned to solution by H_2S treatment, and third, 15.9 mg of soluble N representing 34 % of the total soluble N, which is not precipitated by mercuric nitrate. This latter fraction, containing all of the sugar, has not been analyzed further.

Direct hydrolysis of the mercury precipitate without prior treatment with H_2S yielded the array of amino acids shown in column 2 of table I. Of special note is the presence of proline, hydroxyproline, and cysteic acid, the absence of any basic amino acids, and the absence of aromatic amino acids. Since the presence of mercuric ion during hydrolysis fosters oxidation (for example, cysteine appears as cysteic acid), these chromatographic results are qualitative.

As shown in figure 1, much of the N in the mercury precipitate is liberated on treatment with H_2S, yet direct chromatography of the nitrogenous compounds so liberated shows, as summarized in column 3 of table I, only traces of amino acids, a notable absence of the amides, glutamine and asparagine, and a single, large, intense spot. The latter spot, the position of which is shown in figure 2, reacts slowly with ninhydrin, the color becoming more intense on standing at room temperature for several days. That this spot does indeed represent a peptide is shown by its disappearance on acid hydrolysis with the concurrent appearance of 7 or 8 amino acids. As shown in column 4 of table I, threonine occurs in very small amounts even after acid hydrolysis and it is quite possible that this compound does not occur in the

TABLE I

NINHYDRIN-REACTING COMPOUNDS IN MAPLE SAP AND SYRUP

AMINO ACID	μG/GM COMPOUNDS FROM LYOPHILIZED SAP				μG/ML COMPOUNDS FROM SYRUP	
	HYDROLYSATE #1	HYDROLYSATE #2	SOLUTION #1	HYDROLYSATE #3	SOLUTION #2	HYDROLYSATE #4
Cysteic (from cystine) ...	neg	pos	neg	neg	neg	neg
Aspartic	8.2	pos	trace	3.28	10.8	13.9
Glutamic	10.4	pos	trace	13.1	12.3	16.3
Serine	11.5	pos	trace	0.71	neg	neg
Glycine	4.5	pos	trace	6.55	0.94	3.4
Threonine	5.0	pos	trace	trace	neg	trace
Alanine	8.4	pos	trace	2.26	neg	0.9
Arginine	3.5	neg	neg	neg	neg	neg
Lysine	3.1	neg	neg	neg	neg	neg
Valine	5.9	pos	trace	1.05	neg	0.5
Leucines	19.6	pos	trace	1.43	neg	0.5
Proline	11.5	pos	neg	neg	neg	trace
Hydroxyproline	trace	pos	neg	neg	neg	trace
Peptide	neg	neg	Predominant spot	neg	pos	neg

Hydrolysate #1: Alcohol insoluble fraction.
Hydrolysate #2: Hg precipitate of the alcohol soluble nitrogen.
Hydrolysate #3: Nitrogen compounds liberated by H_2S from the Hg precipitate of the alcohol soluble fraction of sap.
Hydrolysate #4: Nitrogen compounds liberated by H_2S from the Hg precipitate of maple syrup.
Solution #1: Nitrogen compounds liberated by H_2S from the Hg precipitate of the alcohol soluble fraction of sap.
Solution #2: Nitrogen compounds liberated by H_2S from the Hg precipitate of maple syrup.

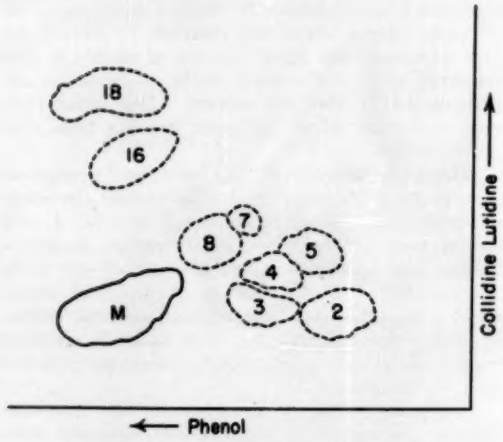

FIG. 2. Two-dimensional chromatogram of the peptide-containing fraction of maple sap. 2. Aspartic. 3. Glutamic. 4. Glycine. 5. Serine. 7. Threonine. 8. Alanine. 16. Valine. 18. Leucines. M. Peptide.

peptide itself but was present originally in trace amounts in the free form.

The absence of proline, hydroxy-proline, and cysteine (as cysteic acid) in this fraction, column 4, table I, coupled with their presence in the hydrolyzed mercury precipitate, indicates that these amino acids occur in a combination which is not liberated by the treatment with H_2S. This observation is entirely reasonable in the light of the well known effect of mercuric and other heavy metallic ions on the sulphydryl group. The nitrogen fraction containing the cysteine is apparently permanently denatured by the treatment with mercuric nitrate.

The data in table II permit an estimate to be made of the minimum chain length of the peptide. The six-fold increase on hydrolysis in the amino N of the fraction containing the peptide is an estimate of the amino groups or amide groups freed from the peptide linkages by the acid hydrolysis. This six-fold estimate is probably low since the ninhydrin activity before hydrolysis contains that amount due to traces of free amino acids as well as that due to the terminal amino groups of the unhydrolyzed peptide itself. Approximate molecular composition of the

TABLE II

EFFECT OF HYDROLYSIS ON THE FREE AMINO NITROGEN OF FRACTIONS FROM MAPLE SAP

NITROGEN FRACTION ANALYZED	FREE AMINO NITROGEN		INCREASE ON HYDROLYSIS
	UNHYDROLYZED	HYDROLYZED	
	μg/gm	μg/gm	%
Freeze dried sap * ..	7.80	45.93	490
Syrup **	19.24	28.4	50

* Alcohol soluble fraction, precipitated with Hg and treated with H_2S.
** Precipitated with Hg and treated with H_2S.

TABLE III

AMINO ACID COMPOSITION OF NITROGEN FRACTIONS FROM MAPLE SAP

	ANALYSIS OF LYOPHILIZED SAP: ALCOHOL SOLUBLE FRACTION, PRECIPITATED BY Hg, LIBERATED BY H_2S, HYDROLYZED		ANALYSIS OF BOILED SAP: PRECIPITATED WITH Hg, LIBERATED BY H_2S, AMINO ACIDS FREED ON HYDROLYSIS	
Amino acid	*μM/100 gm*	*Approx. molar ratios*	*μM/140 ml**	*Approx. molar ratios*
Aspartic	2.46	5.0	3.25	14.0
Glutamic ...	8.91	18.0	3.81	16.0
Serine	0.67	1.0	...	0.0
Glycine	8.73	18.0	4.59	20.0
Alanine	2.54	5.0	1.46	6.0
Valine	0.90	2.0	0.53	2.0
Leucines ...	1.09	2.0	0.48	2.0

* 140 ml of boiled sap = 100 gm dry matter.

peptide as derived from further analysis of the chromatographic data is shown in table III.

In summary, the amino acid complement of the alcohol soluble N precipitated by mercuric nitrate is accounted for qualitatively in two parts, a peptide fraction resolubilized by H_2S treatment, showing 7 amino acids as opposed to the 11 amino acids found after direct hydrolysis of the mercury precipitate.

ALCOHOL INSOLUBLE NITROGEN: The experimental results clearly indicate that maple sap contains only a small amount of alcohol insoluble N. As shown in table I, column 1, chromatography of the alcohol insoluble fraction after hydrolysis shows only a partial array of the amino acids which would be expected if this material were truly protein in nature. Of special interest is the apparent lack of aromatic amino acids in this fraction. Many of the qualitative tests for protein are based on the presence of aromatic nuclei in the protein molecule. This accounts for the apparent failure of others to detect the more complicated nitrogenous compounds in maple sap by the use of the xanthoproteic test, Millon's reaction, or the glyoxylic acid test.

Since the alcohol soluble fraction lacks so many of the amino acids normally considered to be integral parts of the protein molecule, it seems best to be cautious in designating this material as protein; on the other hand, it contains a wider range of amino acids than would normally be expected to occur in a simple peptide. Since no estimate of the molecular weight is available, it seems best to leave open the question of whether or not a very peculiar protein or an equally peculiar peptide or mixture of peptides is present in maple saps.

SYRUP: Certain analyses were made to show the nature of changes occurring in the sap during the boiling process. However, a complete analysis of maple syrup was not carried out.

During boiling of the sap a scum of high N content floats to the top of the solution. Kjeldahl analysis showed this scum to contain 3.8 % N on a dry weight basis, and if corrected for ash, approximately 10.5 % N is present. Chromatographic analysis after hydrolysis showed the presence of the same array of amino acids which was found in the alcohol insoluble fraction of whole sap. Since boiled sap shows no alcohol insoluble N, apparently this so-called proteinaceous material in whole sap is denatured and floats to the surface of the dense, syrupy solution.

Sap concentrated by boiling was made up to a 70 % alcoholic solution and was tested with mercuric nitrate to determine whether any of the peptide present in the sap survived the boiling process. A precipitate formed with the mercuric nitrate reagent and this was then decomposed with H_2S. After removal of the mercuric sulfide, the resulting solution was analyzed chromatographically as was an hydrolyzed aliquot of the same solution. This solution did indeed show an acid unstable spot corresponding exactly to the sap peptide both in position on the chromatograms and in the length of time required for development of the characteristic ninhydrin color. This solution also showed at least three, faint, acid unstable, ninhydrin reacting spots which may represent partial breakdown products of the original peptide. The complete analyses are shown in the last two columns of table I. That some breakdown has indeed occurred is shown by the small increase after hydrolysis of free amino N, measured by the method of Moore and Stein, as shown in table II. The increase in free amino N on hydrolysis is now only about 50 % while in the analogous sap analysis the increase was nearly 500 %. Molar ratios for the amino acids released during this hydrolysis are shown in table III. After boiling the composition of the material precipitable by Hg was different notably with respect to serine, which was not obtained on hydrolysis, and with respect to aspartic acid, which was more conspicuous in the hydrolysate. These data could be interpreted to mean that: (a) the peptide-like complex of the unboiled sap loses serine on boiling; (b) there is some other nitrogenous material in freeze dried sap which is not precipitable until after the boiling process or which may be precipitable but is not returned to solution by H_2S treatment unless previously boiled. This material is richer in aspartic acid than the peptide described from the lyophilized sap.

Clearly the problems concerning the effects of boiling maple sap on the nitrogenous compounds require further study. The starting point for such research can now be the knowledge that the nitrogenous constituents of exuded sap include a peptide-like complex, precipitable by Hg and containing the amino acids in the proportions shown in table III.

DISCUSSION

The chief difficulties encountered in this investigation arose from the fact that the N content of maple sap is very low in comparison with its sugar content. The alcohol soluble fraction of the sap contains nearly 7,500 times as much sugar as N, hence

recourse was taken to the mercuric nitrate precipitation in an effort to separate sugar and nitrogenous compounds. While this separation was not complete, as shown in figure 1, it made possible at least the partial analyses given.

The physiological significance of the nitrogenous compounds found is open to discussion. Certainly, occurring in the xylem sap as they do, they cannot be an active part of the metabolic pool of the plant, though they are doubtless subject to movement, and may well be reabsorbed and utilized by the living cells. On the other hand, there are reports by Bois and Nadeau (1) and Meusse (9) of enzymes occurring in maple sap. Any enzymic material would certainly show up in the alcohol insoluble fraction as protein, yet the analyses given made the identity of this fraction with a true protein rather doubtful.

It is apparent from the data that all of the N is not accounted for in the analyses given. The unaccounted N is not inorganic and remains unexplained. Plouvier (12) and Echevin, Brunel, and Sartorius (3) both found large amounts of allantoin in the green leaves of a variety of different members of the genus Acer, yet qualitative tests (using *p*-dimethylaminobenzaldehyde) failed to indicate this or other ureides in the sap or in the fraction containing most of the easily hydrolysable N. The relatively large amount of easily hydrolysable N suggests the possible presence of amides of organic acids. Although no direct evidence of this exists, the organic acids themselves, especially malic acid, are present in large amounts (11). Porter et al (13) have in fact shown that at least 14 organic acids other than amino acids are present in maple sap. Since their procedure was a chromatographic one, it is not impossible that some of the spots shown could be half-amides of dicarboxylic acids.

Summary

1. Lyophilized sap from the sapwood of sugar maple *Acer saccharum*, contains N in the following known forms:

a. Ammonia
b. Peptides, one of which is liberated from its mercury precipitate by H_2S treatment, and on hydrolysis yields at least seven amino acids; and another or others which are not liberated by H_2S treatment and which yield on hydrolysis at least cysteine, proline, and hydroxyproline.
c. An alcohol insoluble fraction accounting for 17 % of the total N. This fraction is protein-like in its alcohol solubility but does not yield on hydrolysis all of the amino acids which one would expect to find in a normal plant protein.
d. A compound or compounds which yields ammonia on the gentle acid hydrolysis usually used for amide determination. No further evidence that this compound or compounds is an amide has been found.

2. When maple sap is concentrated to syrup by boiling, a scum arises to the surface. Since this scum contains the same array of amino acids found in the alcohol insoluble portion of whole sap and since boiled sap contains no alcohol insoluble N, it is probable that this scum represents the alcohol insoluble N present in sap prior to boiling. During the boiling process, the alcohol soluble peptide is partially broken down to free amino acids and to lower peptides.

3. There is at present no clue to the ultimate origin of these nitrogenous compounds and to their relation, if any, to the problem of sap flow.

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STUDIES IN PLANT METABOLISM. IV. COMPARATIVE EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID AND OTHER PLANT GROWTH REGULATORS ON PHOSPHORUS METABOLISM IN BEAN PLANTS[1,2,3]

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Accumulated evidence has indicated that the physiological effect of 2,4-dichlorophenoxyacetic acid (2,4-D) in plants may function through its effect on processes of respiration (1, 2, 3, 4, 5) as well as on photosynthesis (6). Since the precise mechanism by which plant cells respond to 2,4-D has yet to be elucidated, and the participation of phosphorus in biological oxidation-reduction reactions had been found in both higher animals and microorganisms, an investigation was initiated to study the comparative effects of 2,4-D and other plant growth regulators on phosphorus metabolism in order to clarify, in part, the herbicidal effect of 2,4-D.

The immediate objectives of the work reported here are as follows: the comparative effects of 2,4-D and other plant growth regulators on (1) the phosphorus uptake, (2) the movement and the distribution of phosphorus, and (3) the incorporation of radioactive phosphorus into some intermediate compounds in bean plants.

MATERIALS AND METHODS

GROWTH REGULATOR SOLUTIONS: Immediately prior to use, 95 % ethanol solutions, each containing 0.1 % plant growth regulators (2,4-D acid, indoleacetic acid, indolebutyric acid or α-napthaleneacetic acid) and 0.5 % Tween-20, were prepared and were used throughout this experiment.

TREATMENT OF PLANTS: Bean plants (*Phaseolus vulgaris*, var. Black Valentine) were grown in sand culture under greenhouse conditions unless otherwise noted. The nutrient solution of Biddulph (7) was employed. In several trials, the plants were randomized and divided into four groups. Three groups were treated with 10 μg, 50 μg or 100 μg of 2,4-D per plant on the midrib of one of the primary leaves which was almost fully expanded but whose terminal bud was still small. The fourth served as controls. The same nutrient solution containing 10 to 50 μc P^{32} per liter was then applied daily to the medium in which the plants were growing. All plants were harvested after 7 days, and the roots were carefully washed under running water to remove the unabsorbed radioactive P. All samples were composited, weighed and homogenized in a Waring Blendor with 20 times their weight of water. An aliquot of each homogenate was taken out with a pipette and dried in a 1 inch diameter stainless steel cup under an infra-red lamp. The radioactivities of all samples were measured directly with a thin mica window G-M counter (1.7 mg/cm²). They represent a relative fraction of the total P absorbed and accumulated during the experimental period. Other aliquots were centrifuged with a Servall angle centrifuge to remove the residue and equivalent amounts of the supernatants were dried in the same manner and the radioactivities of these samples which represent the water soluble P were again measured. A typical set of data is shown in table I.

In the study of the effect of 2,4-D and other plant growth regulators on P metabolism, 60 bean plants were grown in the same manner in 2 flats and divided into 6 randomized groups consisting of 10 plants each. The first and fourth groups received no treatment. The second group received 50 μg 2,4-D treatment to each plant (the chemical was applied on one of the primary leaves) and the 3rd, 5th and 6th groups received 250 μg of indoleacetic acid, 250 μg indolebutyric acid and 250 μg α-naphthaleneacetic acid per each plant respectively. A nutrient solution containing 10 μc per l of P^{32} was then given daily to the plants after 24 hours of treatment. Any difference in absorption and distribution of P^{32} in the bean plants and in the incorporation of P^{32} into the P intermediate compounds if found between growth regulator treated and control bean plants would be the result obtained from the physiological effects which occurred after the treatment. All plants were

TABLE I

EFFECT OF VARIOUS AMOUNTS OF 2,4-D TREATMENT ON PHOSPHORUS UPTAKE IN BEAN PLANTS (EACH GROUP CONSISTS OF 5 PLANTS)

	2,4-D Treatment, μg			
	0	10	50	100
	Total P^{32} activity per 25 mg fresh sample, cpm			
Leaf	490	370	190	150
Stem	330	425	350	370
Root	9800	9850	9550	9000
	Water soluble P^{32} activity per 25 mg fresh sample, cpm			
Leaf	348	310	148	115
Stem	295	345	255	282
Root	8050	7570	9300	8800

[1] Received February 8, 1954.

[2] This work was supported by a grant from the Atomic Energy Commission.

[3] Approved for publication as Technical Paper No. 834 by the Director of the Oregon Agricultural Experiment Station. Contribution of the Department of Agricultural Chemistry.

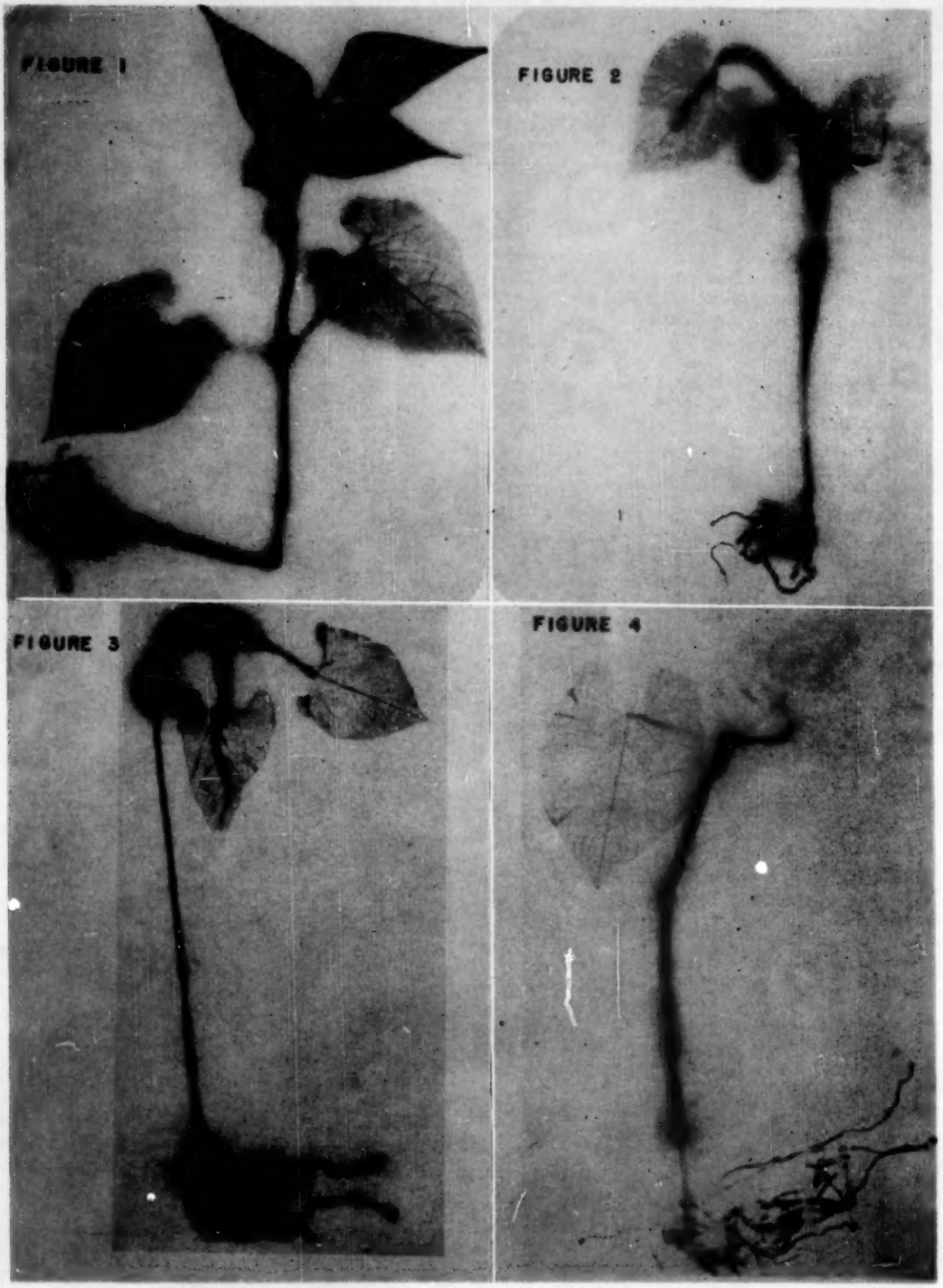

FIG. 1. Radioautograph of control bean plant showing the distribution of P^{32} after a 7-day absorption period.

harvested after 7 days. Two plants from each group were used for making radioautographs to show the distribution of P^{32}. The remaining 8 plants were divided into leaf and stem portions, the root being discarded. The samples were composited, weighed, and homogenized with 20 times their weight of water. The homogenates were decanted into flasks and steamed immediately for 10 minutes to inactivate any enzymes present. An aliquot was centrifuged to remove the residue, and the supernatant was preserved under a layer of toluene and kept in a refrigerator. These samples were subjected later to chemical analyses for inorganic P, organic P, and measurement of radioactivity. Chromatographic separation, identification and determination of the P intermediate compounds of these samples were also performed.

MEASUREMENT OF RADIOACTIVITY: A Tracerlab 64 scaler equipped with a thin mica window G-M counter, or a windowless gas flow counter was used depending upon the degree of activity of the sample. Equal amounts of supernatant solutions from growth regulator treated or from control bean plants were used in every case in order to minimize the error from the self-absorption. No correction for self-absorption was made in any of the measurements. In many instances, an Autoscaler equipped with an automatic sample changer was used for measuring the radioactivity of paper chromatograms. All samples were measured to within 5 % probable error.

RADIOAUTOGRAPHY: The radioautographs were prepared by exposing x-ray film to the plants containing P^{32}, for 22 to 48 hours, then developed with a Kodak D-72 developer.

CHROMATOGRAPHIC SEPARATION: One-dimensional chromatograms of the supernatant fluids were prepared in the usual way. The supernatant liquid was added dropwise with a glass dropper in a small spot to paper strips, about 6 cm from one end of the strip, until the desired amount had been applied. Whatman #1 paper strips (1″ × 22″) were used. The strips were developed with a mixture of isopropyl ether and 90 % formic acid in a volume proportion of 9:6, respectively (8). After development the chromatographic strips were thoroughly dried in air and cut into 1-cm sections, starting from the spot and consecutively numbered. Each section was placed in a stainless steel, cupped planchet and the radioactivity was measured. The relative radioactivity of the spots was then computed as described previously (9).

In other strips, after enough radioactivity from the supernatant liquids had been applied on the strips, known amounts of phosphoglyceric acid, glucose-1-phosphate and hexosediphosphate (about 50 μg each) were also applied to the spot. After development, the chromatograms were sprayed with a perchlorate-molybdate reagent. The technique of Hanes and Isherwood (10) was followed in producing the blue color of the P chromatograms. After recording the positions of P spots, the strips were again cut into 1-cm sections and the radioactivity of each section was measured in the same manner. The locations of blue P spots and the radioactive spots were compared. Several radioactive spots were identified by this process as inorganic phosphate, phosphoglyceric acid, glucose-1-phosphate and hexosediphosphate.

DETERMINATION OF TOTAL AND INORGANIC PHOSPHORUS: The method of Fiske and Subbarow (11) was followed. The color of the solution was measured with a Coleman electric photometer and was compared with standards produced from known amounts of P.

RESULTS AND DISCUSSION

THE EFFECT OF VARIOUS AMOUNTS OF 2,4-D ON THE UPTAKE AND DISTRIBUTION OF P^{32} IN BEAN PLANTS: Data regarding the P^{32} activities per 25 mg fresh samples of leaf, stem and root from the 2,4-D treated and the control bean plants are represented in table I. In all cases, the P^{32} activities of leaves from 2,4-D treated plants were much less when compared to the leaves from the control plants, while the activities in either the stem or root from these two groups of plants showed no significant differences. When 2,4-D treatments were increased from 10 μg to 100 μg per plant, the accumulation of P^{32} activity in the leaf from different treatments decreased accordingly. This observation indicated that 2,4-D treatment affected the upward movement and the supply of P to the leaves. Consequently, the insufficient supply of P in the leaf may directly or indirectly affect both the important photosynthetic and respiratory processes of the plant.

THE EFFECT OF 2,4-D AND OTHER PLANT GROWTH REGULATORS ON THE DISTRIBUTION OF P^{32} IN BEAN PLANTS: As shown in figures 1 to 4, the distribution of P^{32} in growth regulator treated plants differed from that found in the control plants. The radioautograph of the IBA treated plants was quite similar to that of the IAA treated one; therefore it was not included. In general, the P^{32} was concentrated in the petiole of the treated leaf, the midrib of the treated leaf, and in the first internode. The concentration of P^{32} was extended to part of the hypocotyl in α-naphthaleneacetic acid treated plants. The accumulation and the distribution of P^{32} in the bean plants are probably determined by the plant growth regulator. The distribution pattern of P^{32} in 2,4-D treated plants is very similar to the accumulation

FIG. 2. Radioautograph of 2,4-D treated bean plant showing the distribution of P^{32} after a 7-day absorption period. (50 μg of 2,4-D were applied on the left primary leaf.)

FIG. 3. Radioautograph of indoleacetic acid treated bean plant showing the distribution of P^{32} after a 7-day absorption period. (250 μg of IAA were applied on the left primary leaf.)

FIG. 4. Radioautograph of α naphthaleneacetic acid treated bean plant showing the distribution of P^{32} after a 7-day absorption period. (250 μg of NAA were applied on the right primary leaf.)

pattern of 2,4-D (12). This observation may suggest that the distribution of P^{32} is associated with the distribution of the growth regulators. In 1948, Brunstetter et al (13) demonstrated that when bean plants were treated with 3-indoleacetic acid, the P content in the treated stem increased about 5 times over that of the control after 148 hours. Our result showed that the accumulation of P^{32} in IAA treated stems was the same as compared to the control stems. However, the total P content in the treated stems was increased about 60 % over that of the controls and presumably this increase was due to the transport of P from the leaf. Whether or not the physiological effects of 2,4-D and of other growth regulators on bean plants, in regard to the distribution of radioactive P, are the same has yet to be studied. However, the growth regulator treatment will certainly modify the distribution and accumulation pattern of phosphorus in bean plants.

THE EFFECT OF 2,4-D AND OTHER GROWTH REGULATORS ON THE INCORPORATION OF P^{32} INTO SOME PHOSPHORUS INTERMEDIATES: It is obvious that both the total water soluble P^{32} activity and the specific activity decreased in the leaves of all bean plants treated with growth regulators. The effect was more pronounced in 2,4-D treated plants. The total P^{32} activity was found to be greatly increased in 2,4-D treated stems over that of the control, slightly decreased in the IBA treated stem, and without significant changes in IAA- and NAA-treated stems. The percentage of inorganic P was found to be higher in the leaf and lower in the stem of 2,4-D treated plants as compared to the control. Also, the incorporation of P^{32} into glucose-1-phosphate and hexosediphosphate was less in the leaf and greater in the stem of 2,4-D treated plants. A great increase in hexosediphosphate was observed only in the 2,4-D treated stem while no significant, or slight, change was found with the other plant growth regulator treatments. The results demonstrated clearly that all of the growth regulators affected the incorporation of P^{32} into one or more P intermediates. 2,4-D has the greatest effect over the other growth regulators on the incorporation of P^{32} into P intermediates.

SUMMARY

The effects of 2,4-D and of other growth regulators on the P metabolism in bean plants was investigated. It was found that 2,4-D treatments greatly reduced the upward movement of radioactive P to the leaves; the degree of reduction was proportioned to the amount of 2,4-D supplied.

Treatment with 2,4-D, IAA, IBA, or NAA modified the distribution and accumulation pattern of radioactive P in bean plants.

The incorporation of P^{32} into some P intermediate compounds in the water soluble fraction was affected by plant growth regulator treatment. The greatest effect was observed in 2,4-D treated plants.

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PROMOTION BY ZINC OF THE FORMATION OF CYTOCHROMES IN USTILAGO SPHAEROGENA [1]

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Ustilago sphaerogena is a smut fungus which is parasitic on the grass *Echinochloa crus-galli* but may be cultured saprophytically in the sporidial stage of its life cycle. The form, rate of growth, and color of the sporidia were found (29) to be dependent upon the medium in which the fungus is grown. In a medium containing 2 % yeast extract and 2 % sucrose, large, rapidly growing pink sporidia which bud in the manner of yeasts are produced in shake-culture. However, in other organic and inorganic media, filamentous, slower growing, white sporidia are produced. The difference in color is striking and was found (29) to be due to the pigment, cytochrome c. The effect of the nutrient medium on the production of other cytochromes and cytochrome oxidase as well as cytochrome c is presented here. Evidence is given that the substance in yeast extract responsible for the high level of cytochrome in this organism is zinc.

MATERIALS AND METHODS

CULTURE OF THE SMUT: All cultures used were derived from one single-spore isolate of *Ustilago sphaerogena* except in the analyses of cultures of different ages, for which another isolate was used. A 2 % Difco yeast extract medium containing 2 % sucrose was used whenever yeast extract (Y.E.) is designated, unless otherwise described. Modified Czapek's medium and double-strength Henderson-Snell medium (Medium B) (14) without the purines and pyrimidines were used particularly in some of the early experiments. Medium B was later revised to the composition given below,[3] and this revised, synthetic medium is designated as Medium A. All media were sterilized by autoclaving for 15 minutes at 15 pounds pressure.

Cultures were grown in 150 ml of liquid media in 500-ml Erlenmeyer flasks kept on a horizontal shaker in a controlled temperature room at 23 to 25° C. The rate of shaking was 94 cycles per minute, each cycle consisting of two 10.2-cm strokes.

Sporidia were harvested by centrifugation at 4° C for 5 minues at 950 × g after 2 days' growth in a yeast extract medium or 3 days in synthetic media. The cells were washed 3 times with distilled water by re-suspension and re-centrifugation.

MANOMETRIC PROCEDURES: Sporidial respiration was measured at 20° C using conventional Warburg manometric techniques. Suspensions of washed cells containing 3 to 10 mg dry weight of cells and buffered at pH 6.0 with M/30 phosphate were used in each vessel. The concentration of cells was adjusted to fall within this range for each determination, as the Q_{O_2} is affected by cell concentration but is constant within this particular range. A 0.073 M concentration of sucrose was employed as the substrate, although glucose gives similar results. Three-tenths ml of 10 % KOH was used in the center well, and the total liquid volume in the main chamber was 3.0 ml. The vessels were shaken at a rate of 84 strokes per minute, each stroke consisting of two, 7.6-cm arcs. Dry weights were obtained by filtering suspensions through asbestos-lined Gooch crucibles and drying the cells at 85° C for 24 hours.

Sporidia were homogenized by grinding in a mortar with alumina (ALCOA No. A-301). The quantity of alumina used was 1 to 2 times the volume of the packed cells. The mixture was ground until it became fluid, alumina added again to form a thick paste, and the paste re-ground until it again became liquid. The slurry was transferred with 15 ml of 0.22 M sucrose and centrifuged at 978 × g for ten minutes at 4° C. The cell-free supernatant was then used as the enzyme preparation, either directly or after diluting by 1/3 with 0.22 M sucrose. Other methods of homogenization were tried but did not effectively break the cells of this organism.

The cytochrome oxidase activity in homogenates was determined essentially by the manometric method of Schneider and Potter (22). All determinations were made at 20° C in Warburg vessels as described for the measurement of respiration. Cytochrome c solutions were prepared in distilled water from the product of the Sigma Chemical Company, assuming a molecular weight of 16,500. A final concentration of 4×10^{-5} M cytochrome c was used throughout. Sixtieth molar ascorbic acid neutralized with KOH was used as the reductant, and 0.4×10^{-3} M $AlCl_3 \cdot 6H_2O$ and M/15 phosphate buffer at pH 7.0, all final concentrations, completed the system. Four concentrations of homogenate (0.25, 0.50, 0.75, and 1.0 ml) were used and sucrose solution added to give a total volume of 3.0 ml and a final sucrose concentration of 0.073 M.

All data reported here are based on the O_2 uptake during a half-hour period beginning 10 minutes after the ascorbate was tipped in. No appreciable O_2 consumption was observed before ascorbate was added, and a 5 or 10 minute lag followed the addition of ascorbate. A constant rate was then maintained for a half hour after which a decrease in O_2 uptake was observed.

A correction for the autoxidation of ascorbate was made by subtracting a value based on the pH of the vessel contents measured at the end of the run.

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[3] Composition of Medium A in quantities per l: 20 gm sucrose, 3.0 gm $NH_4C_2H_3O_2$, 3.0 gm K_2HPO_4, 0.80 gm $MgSO_4 \cdot 7H_2O$, 0.63 gm citric acid, 80 mg $FeSO_4 \cdot 7H_2O$, 0.14 mg $MnSO_4 \cdot 4H_2O$, 0.02 mg $CuSO_4 \cdot 5H_2O$.

These values were obtained by determining the rate of O_2 uptake in the absence of homogenate. Autoxidation is slight at pH 7.0 or below, but above 7.0 begins to rise, becoming very rapid at pH's above 8.0 O_2 uptake, when corrected by this method and plotted against low enzyme concentrations, usually extrapolates to zero activity at zero enzyme concentration. Assays for ascorbic acid oxidase were negative, and no appreciable O_2 uptake is observed in the absence of added cytochrome c.

Activity is expressed as μl of O_2 per hour per mg dry weight of homogenate (Q_{O_2} homogenate). The method of homogenizing does not permit the calculation of activity on the basis of dry weight of original cells, as only a portion of the cells are broken. The homogenate dry weight was obtained by centrifuging an aliquot for 40 minutes at 15,000 × g. The pellet formed was re-suspended in distilled water, recentrifuged, and the washed pellet then dried for 24 hours at 85° C. One to 4 mg/ml homogenate were obtained.

The increase in rate of O_2 uptake is not proportional to the increase in concentration of the homogenate at higher homogenate concentrations. A decrease in O_2 uptake is sometimes found when 1.0 ml homogenate is used (table II). No attempt was made to eliminate this decrease by means of purification of the enzyme preparation. The Q_{O_2} of homogenate was obtained from the plot of homogenate concentration against the rate of O_2 uptake corrected for autoxidation of ascorbate.

The inhibition of oxygen uptake by 0.001 and 0.0001 M sodium azide and potassium cyanide was calculated from simultaneous determinations at 4 homogenate concentrations, with and without the inhibitor. CO inhibition was determined in the dark at 4 homogenate concentrations with an atmosphere of 95 % CO and 5 % O_2 and compared to a system with 95 % N_2 and 5 % O_2. A light intensity of 100 fc at the manometric vessel level was used to test for light reversal of the inhibition.

Spectrophotometric Procedures: The conventional methods of cytochrome c extraction employing hot or cold trichloracetic acid, 0.1 M phosphate, or 0.1 *N* sulfuric acid fail to extract the pigment from this organism. Plasmolysis, homogenization, and subjection of the sporidia to alternate periods of freezing and thawing also fail to release the cytochrome c. A method was therefore worked out for the quantitative determination of cytochrome c in intact cells. Such a method has a definite advantage over methods involving extraction of the pigment, since it avoids unassessed errors arising from incomplete extraction. Recently, however, Neilands (19) has found that cytochrome c may be extracted from *Ustilago sphaerogena* by raising the pH of the suspending medium to 10. Neilands found that the absorption spectrum of the extracted smut-cytochrome c is very similar to that of animal cytochrome c, but that the fungus pigment has a higher molecular weight and lower isoelectric point than the beef heart preparation.

For the investigations described here, a spectrophotometric method was used. It is based on the difference in absorption between a suspension of cells in which the cytochrome c is reduced and one in which the pigment is oxidized. Reduction is readily achieved by the addition of 20 mg ascorbic acid and 0.05 ml of 0.02 M KCN to 3.0 ml cell suspension containing 15 to 45 mg dry weight of cells. Potassium ferricyanide, 0.05 ml of a 0.02 M solution/3 ml suspension, can be used to oxidize the endogenous cytochrome but only after the suspension has been heated for 1.5 hours at 60° C. Because heating changes the background absorption, a suspension was heated and then separated into aliquots to be oxidized and reduced.

All measurements were made on a Beckman photoelectric quartz spectrophotometer, Model DU, using 1-cm, matched, Corex cells. The measurements were made by determining the percentage transmission of the reduced suspension when an oxidized suspension was used as a blank adjusted to 100 % transmission. The absorption spectrum obtained in this manner with solutions of oxidized and reduced cytochrome c is similar to that calculated by Lundegårdh (16) as the ox/red spectrum. He showed that the height of the absorption bands of cytochrome c, when plotted as the difference between the extinction of the reduced and oxidized forms, varied approximately linearly with the percentage of reduced cytochrome c. Chance (3), using suspensions of microorganisms, demonstrated that the difference spectrum is a valid measure of the pigment content.

In the assay for smut cytochrome c, measurements were taken in the above manner at two wave lengths, 540 and 550 mμ. At the former wave length, absorption of the oxidized and reduced forms is almost equal, and at the latter, absorption of the reduced form is maximal for the visible portion of the spectrum. The increase in absorption at 550 mμ over that at 540 mμ is used as the basis for calculating the concentration of cytochrome c. This increase is referred to here as the relative absorption. The optical density is then calculated as $\log I_0/I$, where I_0 is the intensity of the incident light, in this case the transmission of the oxidized form, or 100 %. I is the intensity of the transmitted light, in this case the lower transmission whose reduction is due solely to the absorption by reduced cytochrome c. I is calculated by substracting from 100 the difference in the percentage transmission at the two wave lengths specified. The relative optical density then becomes: $2 - \log [100 - (\%\ \text{transmission}_{540\ m\mu} - \%\ \text{transmission}_{550\ m\mu})]$.

The relative optical density of solutions of pure cytochrome c determined in this manner is proportional to the concentration of cytochrome c from 4×10^{-5} M to 3×10^{-7} M. Cell suspensions also follow the Lambert-Beer law, except in very dilute solutions (fig 1). Successive additions of aliquots of a solution of pure cytochrome c to a cell suspension also give a linear relationship between relative opti-

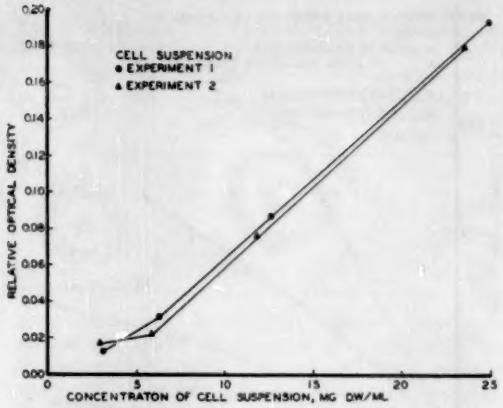

FIG. 1. The relationship between the concentration of endogenous cytochrome c in cell suspensions and the relative optical density.

cal density and the concentration of added cytochrome c.

Using the relative optical density as calculated above, the relative extinction coefficient (a) of cytochrome c was determined from solutions of cytochrome c of known concentration (c) and the diameter of the vessel ($l = 1.0$ cm). From the Lambert-Beer law, $c = \frac{\log I_o/I}{a\,l}$, concentrations of cytochrome c in unknown solutions and in cell suspensions were then calculated using this coefficient. Concentrations of cytochrome c are expressed here as a percentage of the dry weight of the cells. Dry weights were determined as described for the measurement of Q_{O_2}.

SPECTROSCOPIC PROCEDURES: Cytochromes a and b were detected and studied by visual observation of their absorption bands at 605 and 565 mμ, respectively. A diffraction grating spectroscope and a tungsten filament lamp were used for this purpose.

EXPERIMENTAL RESULTS

CYTOCHROME SYSTEM OF CELLS GROWN IN YEAST EXTRACT: The maximal cytochrome c concentration in cells cultured in Y.E. is found after 2 days' growth and in modified Czapek's medium after 3 days (table I). Cells produced in other synthetic media, like those from Czapek's medium, contain maximal quantities of cytochrome c after three days' growth. The isolate used in this particular analysis has a lower and more variable cytochrome c content than the isolate used in the other investigations reported here. Slightly more than 1% Y.E. is required for the production of maximal concentrations of cytochrome c (fig 2).

TABLE I

CYTOCHROME C CONTENT OF CELLS FROM CULTURES OF DIFFERENT AGES

AGE IN DAYS	Y.E. MEDIUM	CZAPEK'S MEDIUM
	% OF DRY WEIGHT	
1	0.08	...
2	0.48	0.03
3	0.43	0.07
4	0.21	0.07
5	0.30	0.05
6	0.22	0.03
7	0.14	0.03

Differences in the levels of other cytochromes are also observed in cells from different media. Spectroscopic examination of cell suspensions from Y.E. reveals, along with the striking absorption bands of reduced cytochrome c at 550 and 520 mμ, faint bands of cytochrome a and b at 605 and 565 mμ, respectively. Suspensions of white cells obtained from synthetic media show no regions of absorption, or, at best, only a faint band of cytochrome c at 550 mμ.

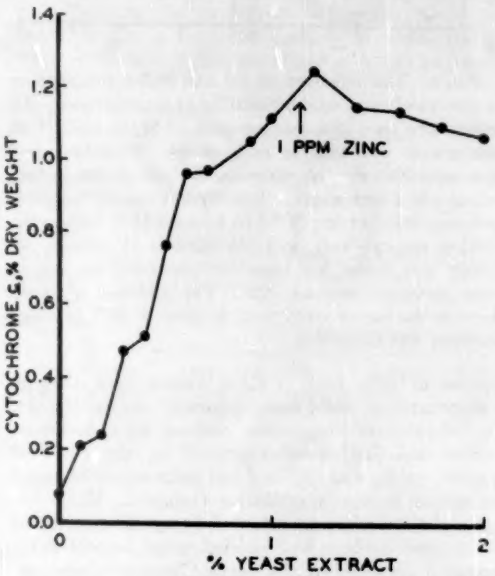

FIG. 2. The influence of yeast extract concentration on cytochrome c production. The concentration of yeast extract which contains 1.0 ppm Zn is indicated by the arrow.

Spectroscopic observation of cells grown in Y.E. also reveals that cytochrome oxidase is present and functional in the intact cell. The band of reduced cytochrome c disappears upon aeration of a suspension and re-appears when aeration is stopped. The activity of the oxidase in homogenates was also investigated. The optimum pH is about 8.0 in phosphate buffer. The effect of pH and buffer composition is shown in figure 3. Because of the high autoxidation above neutrality, activities were determined at pH 7.0 rather than at the optimum pH.

The activity of cytochrome oxidase in homo-

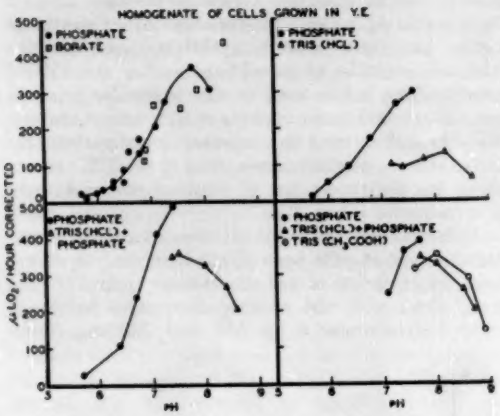

FIG. 3. The influence of pH and buffer composition on the cytochrome oxidase activity of homogenates. All buffers were used at a concentration of M/15, and 1.0 ml homogenate was used in each vessel. Phosphate and boric acid–KCl do not maintain the pH of the system well at pH 8 and above. Tris-(hydroxymethyl)-aminomethane, adjusted to pH 7.0 to 9.0 with HCl, buffers the reaction mixture very well but inhibits O_2 uptake, although this buffer has been recommended for use in some biological systems (13). The addition of phosphate or the use of acetic acid in place of HCl partially abolishes this inhibition.

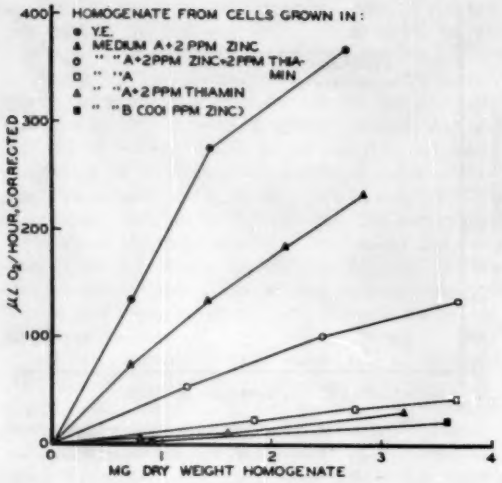

FIG. 4. Cytochrome oxidase activities at varied concentrations of homogenates of cells grown in different media.

genates of cells from Y.E. is higher than that in homogenates of cells from synthetic media (fig 4). The identity of the active enzyme as cytochrome oxidase was further substantiated by the effect of cyanide, azide, and CO and the light reversibility of the carbon monoxide inhibition (tables II, III).

In the experiments reported here, the activity of cytochrome oxidase was studied using animal cytochrome c prepared by the Sigma Chemical Company. The cytochrome c from *U. sphaerogena* is also catalytically active with the cytochrome oxidase of the smut, and the activity is of the same order of magnitude as found with animal cytochrome c.

Despite striking differences in cytochrome oxidase activity of homogenates from cells grown in Y.E. and synthetic media, there are no significant differences in the respiratory rates of intact cells as long as the pH of the nutrient medium is maintained above 4.5 during growth (table V). The Q_{O_2} of the intact cells with sucrose is about 30. Assays for ascorbic acid oxidase, lipoxidase, tyrosinase, and catecholase were negative. The absence of these other oxidases and presence of cytochrome oxidase in homogenates and the observed oxidation-reduction in cells point to cytochrome oxidase as a participant in the respiration of the cells. Despite these facts, this smut, like the fungus *Myrothecium verrucaria* (6), is not inhibited by azide, cyanide, or CO although shown to have a cytochrome oxidase system.

FACTORS RESPONSIBLE FOR CYTOCHROME PRODUCTION: Because of the high levels of cytochrome c produced under favorable conditions, its presence can be detected and the amount estimated roughly by visual observation of the color of packed cells. Using color as a criterion of cytochrome content, the factor in yeast extract responsible for cytochrome c

TABLE II

CO INHIBITION OF CYTOCHROME OXIDASE ACTIVITY OF HOMOGENATES OF CELLS GROWN IN Y.E. PERCENTAGE INHIBITION GIVEN IN PARENTHESES

TIME PERIOD		95% N_2, 5% O_2				95% CO, 5% O_2		
		ML HOMOGENATE				ML HOMOGENATE		
		0.25	0.50	0.75	1.0	0.25	0.50	1.00
		μL O_2/HOUR, CORRECTED FOR AUTOXIDATION						
First 30 min.	Dark	93	136	145	116	3 (95%)	20 (85%)	35 (70%)
Second 30 min.	Light	84	112	118	94	19 (78%)	43 (62%)	83 (12%)
Third 30 min.	Dark	79	101	106	83	1 (99%)	10 (91%)	32 (61%)
Fourth 30 min.	Light	76	95	94	72	19 (75%)	27 (71%)	68 (5%)

TABLE III

CO, CYANIDE, AND AZIDE INHIBITION OF CYTOCHROME OXIDASE ACTIVITY OF HOMOGENATES OF CELLS GROWN IN Y.E.

INHIBITOR	CONCENTRATION OF INHIBITOR	% INHIBITION AT 1.0 MG DRY WEIGHT OF HOMOGENATE
CO–dark	95 %	76
CO–light	95 %	30
KCN	0.001 M	99
KCN	0.0001 M	86
NaN_3	0.001 M	88
NaN_3	0.0001 M	53

production was determined. Initial experiments showed the active substance to be a dialysable, heat stable, ether insoluble component of Difco yeast extract. Neilands (unpublished) observed some formation of cytochrome c in a modified, double-strength Henderson-Snell medium, more than in other synthetic media but less than in yeast extract. This medium contains inorganic salts, sucrose, vitamins, and high concentrations of the 10 essential amino acids. Experiments with this basal medium indicated that the complete amino acid fraction was usually required for cytochrome c production, but sporadic appearance of pink cells was also observed with arginine, isoleucine, and especially methionine. Spectroscopic checks confirmed the presence of cytochrome in these cells. It could not, however, be obtained at will, suggesting the possibility that a contaminating substance was responsible for cytochrome c formation in these cultures. The recognized avidity of amino acids for heavy metals (1) and the release of heavy metals from new glassware (26) further suggested that the contaminant required might be a metallic ion. Tests with dithizone indicated the presence of relatively high concentrations of heavy metals in the amino acid solutions employed. Further credence for this suggestion was found in the observation of Allen (2) that heavy metals other than Fe were involved in cytochrome production by this smut.

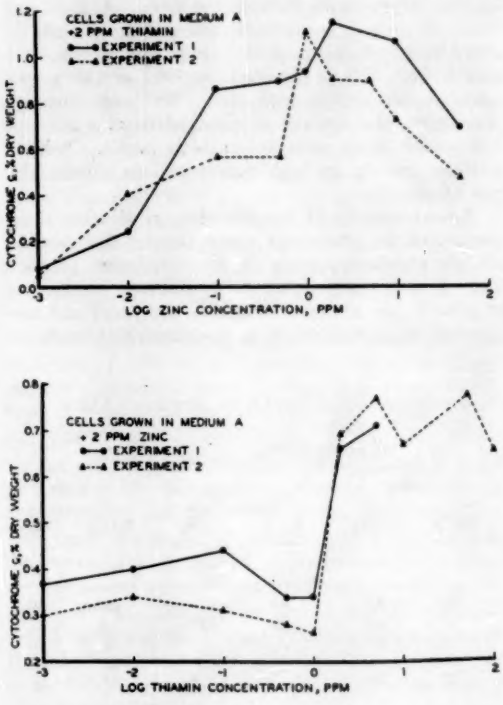

FIG. 5. The influence of Zn and thiamin concentration on cytochrome c production.

The pH of the various synthetic media employed in the preceding experiments dropped rapidly to 2.0 or 3.0 during the growth of the smut. In an attempt to find a synthetic medium in which the pH was maintained during growth at a level comparable to that of yeast extract (pH 5.0 to 6.0), the medium given in footnote 3 was devised. Ammonium acetate was used instead of the nitrate or ammonium compounds of other synthetic media and was found to maintain the pH above 4.5 during growth. Increases in Mn, Cu, and Fe concentrations over those used in previous media did not effect cytochrome c production. However, the addition of higher concentrations of Zn did promote the production of pink cells. Because an effect of the complete vitamin solution had also been noted with the modified Henderson-Snell medium previously employed, vitamins were added to the revised medium. From experiments with vitamins added singly to this revised medium, it was found that thiamin, although ineffective when added without Zn, increased the amount of cytochrome produced in the presence of Zn.

TABLE IV

CYTOCHROME c CONTENT OF CELLS GROWN IN DIFFERENT MEDIA*

EXPT. NO.	Y.E.	MEDIUM A			
		NO SUPPLEMENTS	+2 PPM THIAMIN	+2 PPM Zn	+2 PPM THIAMIN +2 PPM Zn
		% OF DRY WEIGHT			
1	0.78	0.14	0.07	0.45	0.59
2	0.76	0.10	0.06	0.52	1.14
3	0.90	0.03	0.09	0.52	0.90
4	1.06	0.05	0.01	0.44	0.52
5	0.77	0.01	0.01	0.27	0.69
Av.	0.85	0.07	0.05	0.44	0.77

* Y.E. cultures two days old. Other cultures three days old.

One ppm Zn in the presence of thiamin promotes the formation of maximal quantities of cytochrome c (fig 5). Fifty ppm slightly inhibits cytochrome c production, and white cells are again formed when

TABLE V

CYTOCHROME OXIDASE ACTIVITY OF HOMOGENATES OF CELLS GROWN IN DIFFERENT MEDIA

YEAST EXTRACT	MEDIUM A			
	NO SUPPLEMENTS	+2 PPM THIAMIN	+2 PPM Zn	+2 PPM Zn +2 PPM THIAMIN
	Q_{O_2} OF HOMOGENATE			
248	25.5	5.0	98	61
220	18.0	2.0	98	56
190	7.0	..	..	54
187	6.5	..	..	52
170	6.0	..	..	44
169	3.5	..	..	..
156	...	..	..	..
Av. 191	11.1	3.5	98	53
Respiration of cells grown in different media. Values as Q_{O_2}.				
Av. 32	29	29	28	30

the concentration is increased to 100 ppm Zn. The optimal concentration of thiamin for cytochrome c production is 2.0 ppm in the presence of 1.0 ppm Zn (fig 5). Thiamin maintains the pH of the nutrient medium at 6.0 during growth, instead of the usual pH of 4.5, but this effect is observed at a concentration of 0.01 ppm, far lower than the optimal concentration for cytochrome c production. Only white cells are produced if thiamin is added to the basal medium in the absence of Zn, and there is no increase in cytochrome c (table IV). In the revised synthetic medium containing 1 ppm Zn and 2 ppm thiamin, cytochrome c production is comparable to that in a yeast extract medium (table IV). The effect of yeast extract is closely correlated with the concentration of Zn in a yeast extract solution (fig 2).

Spectroscopic observation revealed that cytochromes a and b as well as c are present in cells grown in the nutrient medium with Zn but are not detectable in cultures from low Zn media. The cytochrome oxidase activity of homogenates is likewise higher for cells grown in Zn sufficient media but falls short of that obtained from cells grown in yeast extract (fig 4). Thiamin does not bring about an additional increase, as it does with cytochrome c production, but reduces the activity below that found when Zn alone is added (table V). The inhibition of cytochrome oxidase by cyanide and azide is of the same magnitude in homogenates of cells from synthetic media as from yeast extract media (table VI).

The similarity in the effects of Zn and of yeast extract is not confined to the production of cytochromes but extends to the morphology of the sporidia. Cells grown in Y.E. are ovoid with dense homogeneous contents, whereas those grown in media containing little Zn are filamentous and vacuolate. When 1.0 ppm Zn is added to the basal medium, the cells formed are shorter, wider, less vacuolate, and branch frequently. Although not completely identical, they resemble the cells from Y.E. much more closely than those from media low in Zn. Thiamin, on the other hand, has no effect on cell morphology in either the presence or absence of Zn.

Zn is required for the growth of this smut, but the optimal concentration is 0.001 ppm, far less than the optimal concentration, 1.0 ppm, for cytochrome production. No inhibition of growth is observed at Zn concentrations as high as 100 ppm. A slight increase in growth is observed when thiamin alone is added to the basal medium. Only half of the total growth from Y.E. is obtained from the synthetic medium supplemented with both Zn and thiamin. Maximum total growth is obtained in two days in Y.E. and in three days in synthetic media. Neither thiamin nor Zn in high concentrations affects the rate of growth.

Re-examination of the contribution of other components of the synthetic medium showed that neither Mn nor Cu could replace Zn for cytochrome production. If either is required for cytochrome production or growth, the amounts necessary are small and are supplied as contaminants in the unpurified medium.

TABLE VI

CYANIDE AND AZIDE INHIBITION OF CYTOCHROME OXIDASE ACTIVITY OF CELLS GROWN IN DIFFERENT MEDIA

MEDIUM	CONCENTRATION OF INHIBITOR	ML HOMOGENATE							
		0.25		0.50		0.75		1.00	
		KCN	NaN_3	KCN	NaN_3	KCN	NaN_3	KCN	NaN_3
		% INHIBITION							
Y.E.	0.001 M	100	92	100	90	98	87	99	82
Y.E.	0.0001 M	87	57	87	49	85	37	79	34
Medium B	0.0001 M	...	..	91	77	..	..	76	46
Medium A	0.0001 M	...	..	96	..	..	..	92	43
Medium A +2 ppm Zn +2 ppm thiamin	0.0001 M	...	..	97	76	..	..	93	33

Cu inhibits growth at concentrations greater than 0.25 ppm in the absence of Zn, but in the presence of added Zn, higher concentrations of Cu are required to inhibit. Different samples of both zinc chloride and zinc sulfate were equally effective in promoting maximal cytochrome production.

Although nitrate-, ammonia-, and amino-N are adequate N sources for the growth of this fungus, the ammonium ion is specifically required for cytochrome production. When amino acids or nitrates are employed, only white cells are obtained even though all other conditions for cytochrome production, the presence of Zn and the maintenance of pH above 4.5 during growth, are fulfilled. The presence of acetate serves only to maintain the pH at this level and is not specifically required for pigment production. Pink cells are produced when the pH is maintained above 4.5 by means other than the use of acetate. The maintenance of this pH, however, is necessary for cytochrome production. If a salt such as NH_4Cl is used, the pH falls to 2.0 during growth and little cytochrome is formed.

Synthesis of Cytochromes by Suspensions of Washed Cells: When *Ustilago sphaerogena* is grown in 1 % Y.E. without added carbohydrate, cell masses of a tan color are obtained. No bands of cytochromes a, b, and c appear in suspensions of these cells, but a broad, ill-defined absorption band is found at 555 $m\mu$. When washed cells of this nature are suspended in a medium containing sucrose and phosphate, the band at 555 $m\mu$ disappears and the bands of the normal cytochromes appear within 8 hours. Sucrose and phosphate are the only supplements required for this transformation. When white cells from a Zn-deficient synthetic medium are washed and suspended in a new medium for this period, Zn, ammonium ion, and Fe are required to bring about the formation of the cytochromes. Sucrose and thiamin increase further the amount of cytochrome c formed from such cells. No band at 555 $m\mu$ is observed in the Zn-deficient cells.

Discussion

The high levels of cytochromes produced and the existence of well defined nutrient substances affecting their formation make this organism exceptionally favorable material for studying the synthesis and interrelationships of components of the cytochrome system. Beef heart muscle, generally used for the preparation of this pigment, contains only 191 μg cytochrome c per gm fresh weight of tissue (15). The pink mutant yeast obtained by Ephrussi and Slonimski (25) produces 0.41 % dry weight of cytochrome c, and rat tissues as much as 0.1 to 0.3 % dry weight (7, 21, 27). These values are all considerably lower than the average cytochrome c content of *U. sphaerogena* sporidia grown in Y.E., 0.85 %, or in medium A supplemented with 1 ppm Zn and 2 ppm thiamin, 0.77 %.

Although neither Zn nor thiamin have previously been found to influence the formation of cytochrome or cytochrome oxidase, several other nutrients have been reported to influence their production. Fe is, of course, necessary, and Fe-deficiency has been shown to reduce cytochrome synthesis in yeast (9, 31). Cu increases the level of the cytochrome pigments (5) and of cytochrome oxidase (23) in rat tissues and in yeast (9, 31), but we have found no evidence for a comparable role of Cu in *U. sphaerogena*. Rats fed with glycine-2-C^{14} were shown by Drabkin (8) to incorporate the C^{14} into cytochrome c in the liver. The utilization of glycine in hemoglobin synthesis in duck blood has been shown by Shemin and Wittenberg (24). They found that the entire porphyrin was synthesized from acetate and glycine through a tricarboxylic acid intermediate. In *U. sphaerogena*, however, glycine cannot replace the ammonium ion required for cytochrome formation. Neither glycine nor acetate enhance cytochrome production in the smut, although they support good growth of white sporidia in the absence of other N and C sources. Tipton (28) found no decrease in cytochrome oxidase level in thiamin deficient rat tissues.

The production of cytochrome is not controlled merely by nutrient conditions, however. Ephrussi and Slonimski (10) showed that O_2 was necessary for cytochrome formation in yeast and that the production of cytochrome oxidase depended upon the presence of the appropriate cytoplasmic particles (25). Chen, Ephrussi, and Hottinguer (4) described the genetic mechanism for the inheritance of the ability to produce components of the cytochrome system. In insects, Williams (30) has shown a correlation between hormone concentration and cytochrome oxidase level during metamorphosis, and Tipton (28) and Drabkin (8) have found that thyroxine administration increased the level of cytochrome oxidase in rat tissues.

In *U. sphaerogena*, however, Zn is the limiting factor for cytochrome production. The function of the high cytochrome oxidase and cytochrome pigment level resulting from the addition of Zn is not clear. These high levels do not provide for a higher rate of respiration, which is of the same magnitude in both pink and white cells. If the greater concentrations of cytochrome and cytochrome oxidase are of value to the organism, it must therefore be in some function other than respiration, possibly connected with the parasitism of the fungus.

There have been several reports of Zn affecting the synthesis of other enzymes. Nason (17) found that Zn deficiency in Neurospora inhibited the condensation of serine and indole to form tryptophane, but tryptophane cannot replace Zn in *U. sphaerogena*. Aldolase (20), alcohol dehydrogenase (18), and pyruvic oxidase (12) have all been reported to decrease or disappear entirely from Zn deficient organisms.

In none of the above examples is the specific site of action of Zn known. In *U. sphaerogena*, Zn is most probably concerned in the formation of the porphyrin nucleus, because the level of several of the

cytochromes is increased. The importance of Zn in carbohydrate metabolism in fungi (11) and the role of an intermediate of the tricarboxylic acid cycle in porphyrin formation are also consistant with this hypothesis. However, no direct evidence is available which would exclude an action in the formation of the protein moiety of the cytochromes or in the incorporation of iron into the porphyrin nucleus.

Summary

1. A spectrophotometric method for the quantitative determination of cytochrome c in suspensions of intact cells is described.

2. The sporidia of *U. sphaerogena* grown in 1 % Y.E.–1 % sucrose medium are pink. The color is due largely to cytochrome c, which may constitute as much as 1.0 % of the dry weight of cells in this medium.

3. Sporidia grown in a simple synthetic medium, such as Czapek's or medium B, are white and contain a maximum of 0.08 % cytochrome c, although the yield of cell material is comparable with that obtained in a Y.E. medium and the Q_{O_2} of the cells is similar to that of the pink cells.

4. Y.E. also enhances the production of cytochromes a and b and of cytochrome oxidase. The oxidase has a pH optimum at about 8.0, and is catalytically active in oxidizing the cytochrome of smut sporidia in intact cells and in homogenates.

5. The substance in Y.E. responsible for high cytochrome production is Zn. It cannot be replaced by Fe, Cu, or Mn. Although only 0.001 ppm Zn is required for optimal growth, 1.0 ppm is optimal for pigment formation, which can be obtained in a completely synthetic medium.

6. The addition of 2.0 ppm thiamin results in a further increase in cytochrome formation in the presence of 1.0 ppm Zn, but thiamin is without effect in the absence of Zn and is not a required growth factor.

7. Cytochromes a and b and cytochrome oxidase are also increased by the addition of Zn to a synthetic medium, but not to the high levels produced in Y.E. Their production is not affected by thiamin.

8. The effect of Y.E. upon cell morphology is partially, but not completely, reproduced by Zn.

9. Ammonium ion is specifically required for the enhanced production of cytochrome c in the presence of Zn and thiamin. It cannot be replaced by nitrate or amino acids, although these compounds will serve as adequate sources of N for growth.

10. High cytochrome production requires that the pH be maintained above 4.5 during growth of the fungus and was achieved by the use of ammonium acetate.

11. Suspensions of washed cells from a medium containing Y.E. but no additional carbohydrate are low in cytochrome but will produce cytochrome if supplied with sucrose and phosphate. Similar cells from a Zn-deficient synthetic medium require Zn, ammonium ion, and Fe for cytochrome formation.

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AUXIN PHYSIOLOGY IN BEAN LEAF STALKS[1]

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Auxin production, auxin transport (8), and auxin inactivation (12) in tissues with relatively high auxin concentration have been extensively investigated and reviewed. Little attention, however, has been given to the auxin physiology of tissues with low auxin concentration. This paper reports an investigation of conditions associated with the low auxin concentration found in bean leaf stalks, a concentration of 0.4 μg equivalents of IAA per kg fresh weight (10).

In the authors' opinion, the extension of the term "auxin" to include all plant hormones, as well as all exogenous plant regulators (Zimmerman & Hitchcock, 19), ignores known differences between hormones and known differences between hormones and exogenous plant regulators, and is therefore confusing rather than helpful. In this paper the term "auxin" will be restricted to compounds manufactured by the plant (endogenous) which produce curvature in the standard Avena coleoptile assay. Synthetic compounds (exogenous) will be specifically named; indoleacetic acid will be designated by its abbreviation, IAA. Inactivation is considered a chemical alteration of a compound so that it no longer produces its characteristic chemical or biological reactions. An inactivator is a substance or biochemical system capable of inactivating a specific compound. Inhibition is a reduction or retardation of a response. An inhibitor is a substance which inhibits.

The material used in this investigation consisted of stalks bearing trifoliate leaves of greenhouse-grown Black Valentine beans. The leaves were in all cases fully expanded and showed no sign of senescence. Auxin was determined by the standard Avena coleoptile curvature assay (18), and IAA equivalents were calculated by the formula of van Overbeek (16). The blocks used in all experiments were 1.5 % agar, $2.7 \times 2.7 \times 1.0$ mm. Twelve blocks were used in each part of each experiment and each experiment was repeated at least once. The results given are based on the average curvature of 12 coleoptiles. Other details of method are included in the description of the experiments where they were used.

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Experiments

Auxin Production: No change in auxin concentration from the time the leaf is fully expanded until it is senescent (10) has been found in previous studies with bean leaf stalks on the plant. But a change in auxin concentration in excised bean leaf stalks was found in experiments here reported. In an experiment using three 35-gm lots of freshly cut bean leaf stalks, one lot was frozen, lyophilized, and its auxin extracted with ether. A second lot was submerged in water. The third lot was placed in air in a moist chamber. At the end of 24 hours the second and third lots were treated as the first had been, and their auxin extracted. The details of the method by which these extracts were obtained have already been described (10). The Avena assays of these three lots, reported as microgram equivalent of IAA per kilogram fresh weight, showed at the start of the experiment (first lot) an auxin concentration of 0.4 μg/kg; after 24 hours in air (second lot) a concentration of

0.0 μg/kg; and after 24 hours in water (third lot) a concentration of 1.5 μg/kg.

This accumulation of auxin in excised stalks under water suggests that stalks on the plant produce auxin but either inactivate it or transport it from the stalk. The fact that auxin accumulated in stalks submerged in water but did not accumulate in stalks in air suggests that an oxidative auxin inactivating mechanism is present in bean leaf stalks.

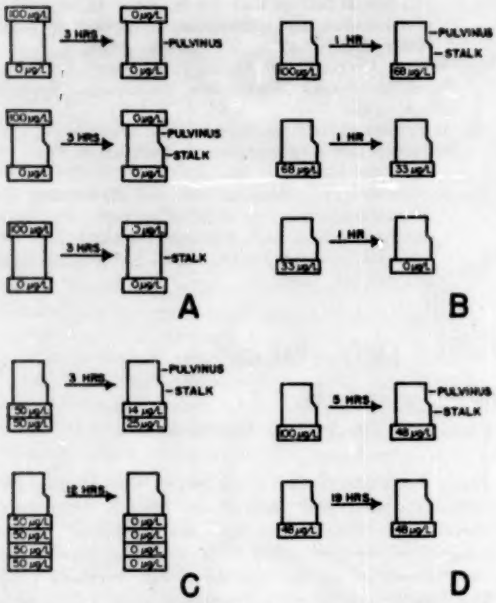

FIG. 1. A. Summary of a 3 hour experiment on transport of IAA by bean leaf stalk sections. A block of agar containing 100 μg/l of IAA was placed on the upper surface of the section and a block of plain agar was placed on the lower, basal, surface. IAA disappeared from the upper blocks but did not appear in the lower blocks. B. Summary of a 3 hour experiment showing three stages in the disappearance of IAA from agar blocks in contact with leaf stalk sections. C. Summaries of a 3 hour, and a 12 hour experiment, showing disappearance of IAA from agar blocks in contact with leaf stalk sections. D. Summary of a 24 hour experiment showing disappearance of IAA from agar blocks in contact with leaf stalk sections that had been boiled. About 50 % of the IAA disappeared in the first 5 hours, but there was no further disappearance in the subsequent 19 hours.

INDOLEACETIC ACID TRANSPORT: The following experiment was designed to test transport of IAA through the leaf stalk: From the leaf stalk subtending the terminal leaflet, 3-mm sections were cut from (a) the leaflet pulvinus, (b) the leaflet abscission zone (since the abscission zone is less than 1 mm long this section included tissues of the leaflet pulvinus and stalk proper), and (c) the stalk 3 cm proximal to the abscission zone. Each section was placed on a block of agar. A similar block containing 100 μg/l of IAA was placed on top of each section. At the end of 3 hours all the blocks were assayed on Avena coleoptiles. The experiment and its results are diagrammed in figure 1 A. None of the blocks produced any response from coleoptiles. Thus this experiment produced no evidence of transport of IAA, but did produce evidence of the presence of an IAA inactivating mechanism in the leaf stalk, since IAA disappeared from the upper blocks in contact with stalk sections for 3 hours.

A similar experiment was conducted with stalk tissues boiled in water for 5 minutes. As in the preceding experiment, after 3 hours no IAA was found in the lower blocks, but in contrast with the preceding experiment, at the end of the 3 hours the upper blocks on each of the 3 types of sections, pulvinus, abscission zone, and stalk, proper, contained respectively, 40, 18, and 14 μg/l of IAA.

This indicates that boiling interfered with the disappearance of IAA but did not completely prevent it.

DIFFUSION EXPERIMENTS: To learn more about the disappearance of IAA, diffusion experiments were performed. In the first of these experiments 3-mm sections of leaf stalk were placed on agar blocks containing 100 μg/l of IAA. In this and subsequent experiments the sections were taken at the abscission zone and therefore contained pulvinal tissue as well as tissue of the stalk proper. The basal, stalk surface was always placed in contact with the agar blocks. The blocks were assayed 3 times at hourly intervals. The results are presented in figure 1 B. They indicate that the disappearance of IAA proceeded at a uniform rate, about 33 μg/l per hour.

In the next experiment the sections were placed on columns of 2 and 4 agar blocks, the agar containing 50 μg/l of IAA. The columns of 2 blocks were assayed at the end of 3 hours and the columns of 4 blocks at the end of 12 hours. The experiment and its results are summarized in figure 1 C. The results indicate that an active diffusion was associated with the disappearance of the IAA.

To learn whether or not enzymes were involved in the disappearance of IAA, sections were boiled in water for 5 minutes and placed on agar containing 100 μg/l of IAA. The blocks were assayed at the end of 5 and 24 hours. The experiment and its results are summarized in figure 1 D. They show a disappearance of approximately 50 % of the IAA in the first 5 hours, with no further disappearance during the next 19 hours. These results indicate that a heat labile mechanism, probably enzymatic, is partly responsible for the disappearance.

To learn of the O_2 relations of the disappearance, sections were placed on agar blocks containing 100 μg/l of IAA and these were then placed in an atmosphere of N_2. The blocks were assayed 5 and 24 hours after being placed in the N_2. The experiment and its results are diagrammed in figure 2 A. After 5 hours, 44 % of the IAA had disappeared. No further loss had occurred at the end of 24 hours. These results

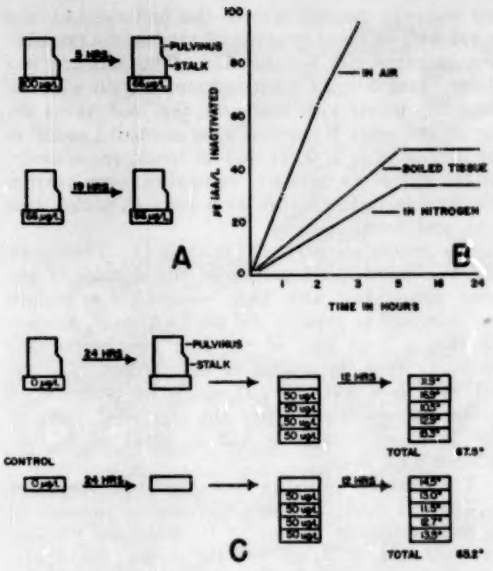

FIG. 2. A. Summary of a 24 hour experiment showing disappearance of IAA from agar blocks in contact with bean leaf stalk sections in an atmosphere of N_2. As with boiling, the disappearance was not complete. B. Summary of the rates of disappearance of IAA from agar blocks in contact with bean leaf stalk sections in air, after boiling, in N_2. C. Summary of an experiment to determine whether an IAA inactivator or inhibitor was diffusing from bean leaf stalk sections. The experiment gave no evidence of diffusion of inactivator or inhibitor.

suggest that O_2 is required for at least a part of the disappearance.

A comparison and summary of the rates of IAA inactivation in blocks in contact with fresh tissue in air, and in N_2, and in blocks in contact with boiled tissue, are shown in figure 2 B.

The disappearance of IAA from the agar blocks in contact with leaf stalks can be interpreted as the result of one, or a combination, of the following actions: a) IAA diffused into the tissue of the section and was inactivated or bound. b) An IAA inactivator diffused from the tissue into the agar and there reacted with IAA. c) An Avena curvature inhibitor diffused from the tissue into the agar. There may be other hypotheses, but the above three are the obvious ones in the light of our present knowledge of auxin physiology.

The effect of boiling in reducing the disappearance of IAA suggests that enzymatic reactions are involved. The similar effect of N_2 suggests that an oxidative reaction is required for the production of the inactivator or inhibitor.

A test for a diffusible inactivator or inhibitor was made in the following experiment. Sections of stalk were placed on blank agar blocks and the combinations kept in a moist chamber for 24 hours. At the end of this period the stalk sections were removed and each of the agar blocks was placed on a stack of 4 similar blocks each of which contained 50 μg/l of IAA. These stacks of 5 blocks each were kept in a moist chamber for 12 hours. All blocks were then assayed. A control was run in an identical manner except that no sections of stalk were used. The experiment and its results are summarized in figure 2 C. In both stacks curvatures from each of the 5 blocks were approximately equal. The total curvature was the same from experimental and control stacks: 67.5° and 65.2°, respectively. Thus this experiment gave no evidence that either an inactivator or an inhibitor diffused from stalk to agar.

Considered together, the results of the diffusion experiments indicated that the disappearance of IAA was due to its diffusion into the sections. Since under ordinary circumstances the disappearance was complete, the IAA was probably bound or chemically changed within the sections.

NATURE OF INACTIVATED INDOLEACETIC ACID: It was not within the scope of this investigation to determine the chemical reactions associated with the disappearance of IAA; however, the results of two simple tests are included here because they may be of interest to other investigators.

Thirty leaf stalks, each about 3 cm long, were placed in a sintered glass filter and covered with 50 ml of a solution of 10 mg/l of IAA in water. Pure O_2 was passed from below through the solution. The solution was tested at the start, after 12 hours, and after 24 hours, with the ferric chloride test for IAA and the Hopkins-Cole test for indole. The ferric chloride reagent was composed of 15 ml of 0.5 M $FeCl_3$, 500 ml distilled water, and 300 ml concentrated H_2SO_4, specific gravity 1.84 (14). Eight ml of this reagent and 2 ml of the IAA solution were placed in a test tube and the color was allowed to develop for 30 minutes. In the Hopkins-Cole test (6), 2 ml of the IAA solution were placed in a test tube and 2 drops of saturated $CuSO_4$ and 6 ml of concentrated H_2SO_4 were added in that order. The color was allowed to develop for 15 minutes. Measurements were made on an Evelyn photoelectric colorimeter using filter No. 515 for the ferric chloride test

TABLE I

EFFECT OF BEAN LEAF STALKS ON A SOLUTION OF IAA AS INDICATED BY THE FERRIC CHLORIDE AND HOPKINS-COLE TESTS

TEST	RESULTS AS MG/L OF IAA			PERCENT DISAPPEARANCE IN 24 HOURS *
	0 hr	*12 hr*	*24 hr*	
Ferric chloride (for IAA) ...	10	6	0	100
Hopkins-Cole (for indole) ..	10	9.5	9.5	5

* Tissueless controls showed no change after 24 hours.

and filter No. 765 for the Hopkins-Cole test. The results are presented in table I. At the end of the 24 hour period, the ferric chloride test for IAA was negative, the Hopkins-Cole test for indole was positive.

These results are consistent with those in the preceding section if they are interpreted as indicating that the IAA diffused into the stalks, was there inactivated, and the inactivated molecule diffused back to the solution.

Inhibition of Coleoptile Curvature: Inhibitors of the coleoptile curvature response have been found in ether extracts of plant tissues by a number of investigators (1, 3, 4, 5, 7, 9, 13). Since the type of inhibitor most frequently found has been non-acid, two experiments were conducted using the Boysen Jensen method which purifies the crude extract by the elimination of non-acid substances. If these eliminated non-acid substances include inhibitors, the resulting purified extract should give a curvature greater than that given by the crude extract. In neither experiment did the purified extract give a curvature greater than that given by the crude extract. Therefore it is assumed that non-acid inhibitors were probably not present in the ether extract of the bean leaf stalks, and the results of the described experiments were probably not due to inhibition of the coleoptile response.

Direct Enzymatic Inactivation of Indoleacetic Acid: Tang and Bonner (14, 15) have described an IAA-inactivating enzyme from etiolated tissues. To determine whether such an enzyme was present in (green) bean leaf stalks, the following experiments were conducted. Stalks were ground in a Waring Blendor with a minimum amount of water. The resulting mixture was filtered through a coarse filter paper and the filtrate, buffered at pH 6.5 with MacIlvain's standard citrate-phosphate buffer, used as an enzyme preparation. Part of this preparation was kept in boiling water for 5 minutes, added to an equal volume of agar (mixture No. 2, table II), and assayed on Avena coleoptiles. A standard solution (200 μg/l) of IAA was buffered as above, part of it mixed with agar as above (mixture No. 1, table II)

Table II

The Effect of a Preparation of Bean Leaf Stalk Enzymes on IAA

	Mixtures	Avena curvature (degrees) with standard deviation	Percent inactivation
No. 1	Buffer and IAA	9.1° ± 0.41	..
No. 2	Buffer and boiled enzyme preparation	0.0°	..
No. 3	Buffer, enzyme preparation and IAA		
	0 hr.	4.1° ± 0.46	53
	1 hr.	4.0° ± 0.41	54
	2 hr.	5.3° ± 0.56	39
	3 hr.	5.2° ± 0.43	43
	24 hr.	4.8° ± 0.67	47
	48 hr.	4.5° ± 0.57	50

and assayed. Another part of this buffered IAA was mixed with an equal quantity of the enzyme preparation (mixture No. 3, table II). This mixture was divided into 6 equal portions: one portion was immediately mixed with agar as above, and Avena assayed; the other 5 portions were incubated at 25° C for periods of 1, 2, 3, 24 and 48 hours, respectively. At the end of its period of incubation, each portion was kept in boiling water for 5 minutes, mixed with agar, and Avena assayed.

The results are presented in table II. They show that the unincubated portion of the mixture of enzyme preparation with IAA produced a curvature less than half as great as did the IAA alone, indicating that a large part of the IAA disappeared immediately upon the making of the mixture. The results also show that time and incubation produced no further disappearance since the incubated portions also produced curvatures half as great as did the buffered IAA alone.

The mixtures tested in this experiment had been kept in test tubes, in which only limited amounts of O_2 were available. In order to determine whether this limitation of O_2 affected the results, the experiment was repeated with O_2 bubbling through the mixtures. The results were the same. There was an initial inactivation, but no increase with time.

These results indicate, as did previous results, the presence of a limited amount of a heat stable substance which is either an IAA inactivator or an inhibitor of the coleoptile response. Since there was no loss of IAA during incubation, a functional IAA-inactivating enzyme was not present.

Discussion

The results of this investigation have emphasized some important aspects of auxin physiology and have led to a tentative conclusion concerning the mechanism of auxin inactivation in bean leaf stalks. The investigation was necessarily conditioned by the methods at present available for the investigation of auxin physiology. These methods are so restricted that it is difficult if not impossible to reach unequivocal conclusions. There is also a serious difficulty in interpretation of Avena assays of ether extracts: the possibility of the presence of Avena coleoptile curvature inhibitors. Such inhibitors are far more widespread than once was realized (1, 3, 4, 5, 6, 9, 13). Some inhibitors have been identified chemically, but most have been described merely as "acid" or "non-acid." A few are characterized by the production of a positive curvature (instead of the usual negative curvature) in the Avena coleoptile. However, neither in this nor in a previous investigation (10) has any evidence of the presence of an inhibitor been found; no positive curvatures appeared, nor any other evidence of either a non-acid inhibitor or a diffusible inhibitor. This evidence indicates that the data obtained with the Avena assays of bean leaves are valid.

Auxin production is frequently assumed to be restricted to young tissues, although this assumption is not supported by the literature (18). In the guayule

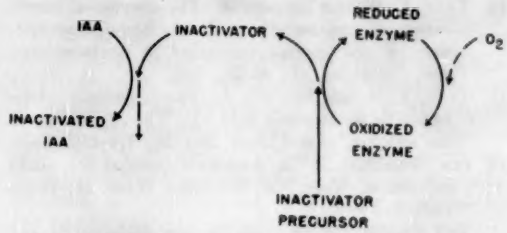

FIG. 3. Diagram of a tentative system of IAA inactivation in bean leaf stalks.

plant, the mature leaves were found to yield the highest concentration of auxin (Smith, 11). In our experiments evidence was obtained that the mature bean leaf stalks produce auxin. Excised bean leaf stalks kept under water showed an increased auxin concentration, whereas excised stalks kept in the air showed no auxin, at the end of the experiment.

The simplest explanation of this result assumes the presence in the bean leaf stalk of both auxin producing and auxin inactivating systems, the auxin producing system having a low O_2 requirement and the auxin inactivating system having a high O_2 requirement. Similar assumptions have been made by Van Raalte (17) to explain the increased diffusion of auxin from Vicia root tips under conditions unfavorable for respiration.

Auxin transport probably occurs in the bean leaf stalk although no evidence for it was obtained in this investigation. Retardation of abscission at the proximal end of a debladed bean leaf stalk has been found to follow the application of IAA to the distal end of the stalk (Addicott & Lynch, 2). This can be interpreted as evidence of the transport of IAA through the stalk. It is probable that failure to obtain transport in this investigation was due largely if not entirely to inactivation. In this connection it is of interest to note that in a recent paper on IAA transport in the bean hypocotyl the low recoveries were explained solely in terms of variations in polarity of IAA transport (Jacobs, 8). The low recoveries could have been explained equally well in terms of inactivation of IAA, or the release of an Avena inhibitor. Unfortunately the data presented do not permit a choice among these alternatives.

Inactivation appears to be the most reasonable explanation of the observed disappearance of auxin and of IAA. Systems for the oxidative inactivation of auxin and IAA are widespread (Steeves et al, 12). This investigation showed auxin disappearance from excised bean leaf stalks under aerobic conditions, and accumulation under anaerobic conditions. IAA was inactivated when in contact with bean leaf stalks and when added to a preparation of bean leaf stalk protoplasm.

Taken together, the evidence indicates that a thermostable inactivator, which can react with auxin and with IAA, is present in bean leaf stalks. The amount of this inactivator present at any one time appears to be limited, since only limited amounts can be demonstrated in tissue that has been boiled or kept in N_2; for continued inactivation, living tissue and O_2 are required. The simplest explanation of this requirement is that an oxidase is necessary for the production of the inactivator. A scheme of the mechanism which is suggested by this evidence is shown in figure 3. Study of this mechanism will be continued; further evidence may alter the present picture.

SUMMARY

Auxin production was found in excised bean leaf stalks when immersed in water. Auxin disappeared from excised bean leaf stalks when they were kept in moist air.

The low auxin concentrations found in previous Avena assays of bean leaf stalks appear to be real; no evidence was found of inhibitors modifying the response of the Avena coleoptile.

IAA transport through sections of bean leaf stalks was not obtained, although IAA disappeared from agar blocks in contact with leaf stalk sections. The disappearance was apparently due to the diffusion of IAA into the sections and its inactivation there.

Evidence of an IAA inactivating system in bean leaf stalks was obtained. The system appears to be composed of at least two parts. In the first part there is oxidative, enzymatic production of an IAA inactivator which is thermostable. In the second part there is a reaction of the inactivator and IAA to form a product which is inactive in the Avena assay. The product is negative to the ferric chloride test (for IAA) but is positive to the Hopkins-Cole test (for indole).

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INCORPORATION OF RADIOACTIVE GLUTAMIC ACID INTO THE PROTEINS OF HIGHER PLANTS

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It has now been demonstrated that enzymes associated with plant mitochondria are able to utilize the respiratory energy conserved by the mitochondria in adenosinetriphosphate (ATP) (3) for the incorporation of glutamic acid into the amide, glutamine (9), and the peptides, γ-glutamylcysteine (11) and glutathione (10). During the course of these investigations, it was noted that mitochondria incorporate radioactive amino acids into their own protein (12). The incorporation process resembles that of peptide bond synthesis in its dependence on ATP. This suggests that further understanding of protein turnover in plants might be obtained by systematic study of the incorporation of labeled amino acids into plant proteins. The only previously recorded observations on radioactive tracer incorporation into plant protein have been those of Arreguin et al (1), using C^{14}-acetate, Racusen and Aronoff (7) with $C^{14}O_2$, and Boroughs and Bonner (4) with C^{14}-leucine and -glycine. In the first two cases mentioned, the substances used were probably converted to amino acids before incorporation into protein. The present investigation was concerned chiefly with establishing some of the overall characteristics of the process of amino acid incorporation in plants. Glutamic acid was utilized for these initial studies as previous results (12) indicated that it is incorporated into protein at relatively high rates even in a cell-free system.

EXPERIMENTAL

The seedlings used in the present studies were germinated and grown in the dark at 25° C as described previously (10). Certain of the mature tissues employed were obtained from local markets. The sectioned tissues were incubated in 0.05 M potassium phosphate buffer (pH 6.8) at 25° C with 1 to 100 microcuries (μc) of either 0.01 M potassium glutamate-2-C^{14} or 0.01 M uniformly labeled potassium glutamate-C^{14}. The amount of radioactivity employed was varied according to the needs of the separate experiments. At the end of the incubation period, the tissue sections were washed free of external amino acid in running water and ground in 0.05 M potassium phosphate buffer (pH 7.5) containing 0.45 M sucrose. Total activity incorporated into the cellular constituents was determined directly from the specific activity of the homogenate. Total protein was precipitated by addition of an equal volume of 12 % trichloroacetic acid at 1° C for 3 hrs. Cellular fractions were separated by centrifugation of the sucrose-phosphate homogenate as described previously (9). Glutathione formation was determined by radioactive assay on the material isolated in the form of cuprous glutathione as earlier described (10). Glutamic acid, α-ketoglutaric acid, and γ-aminobutyric acid were separated chromatographically and the relative activities of the acids estimated directly from that of the glutamic acid.

Radioactive assays were carried out by standard techniques using a thin mica window Geiger-Müller tube and scaling circuit. Protein was washed with trichloroacetic acid, with dilute alkali, and with thioglycollic acid before radioactive assay in order to remove any amino acid either adsorbed or bound through sulfhydryl linkage. These treatments generally removed 10 to 15 % of the total activity. The remainder of the activity was released only by hydrolysis of the protein.

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Results

Transport of Glutamic Acid into Plant Cells: The transport of glutamic acid into the cells of plant tissues, like the transport of metal ions and of carbohydrates, is dependent either directly or indirectly upon respiratory energy. As is illustrated in table I, the uptake of glutamic acid and its accumulation either as glutamic acid or as metabolic products of glutamic acid, is strongly inhibited by the respiratory inhibitors, hydrogen cyanide and hydrogen azide. Anaerobiosis likewise causes significant inhibition of accumulation. The marked inhibition of glutamic acid accumulation caused by dinitrophenol is of particular interest, as Bonner and Millerd (3) have shown that this substance acts as an inhibitor of oxidative phosphorylation in plants. They (3) likewise established ATP to be the product of oxidative phosphorylation in plants. It would seem, therefore, that the inhibitions of accumulation observed here are due to a decreased supply of the ATP that is apparently necessary for accumulation to occur. The lesser degree of inhibition obtained with anaerobiosis would be in agreement with this view, as it is known that small quantities of ATP are produced during glycolysis. Preliminary experiments with several other amino acids (aspartate, glycine, histidine, cysteine) indicate that the accumulation of these amino acids also is dependent upon the availability of respiratory energy.

Table I

Effect of Inhibitors of Respiration and Oxidative Phosphorylation on the Accumulation of C^{14} from Glutamate by Several Plant Tissues *

Addition to system	Total radioactivity in the tissue (cpm) **		
	Bean hypocotyl sections	Carrot root slices	Potato tuber slices
None	2863	3800	1651
Hydrogen cyanide	1417	1318	801
Hydrogen azide	1220	1315	785
Nitrogen atmosphere	2126	3056	1023
Dinitrophenol	1088	889	730

* Sections were incubated for 1 hr at 25° C. Concentration of all inhibitors used was 0.001 M. The incubation medium in each case contained 0.01 M potassium L-glutamate with a total activity of 1 μc. Approximately 0.1 % of the C^{14} added as glutamate was assimilated by the controls.

** cpm = counts per minute.

Metabolism of Absorbed Glutamic Acid: Glutamic acid is apparently metabolized at a rapid rate in living plant cells. Previous studies (10, 11) have demonstrated that it is readily coupled with certain other amino acids to form simple peptides. Table II gives a balance sheet for the fate of glutamic acid in a short time experiment. It can be seen that the glutamic acid has entered into both degradative and synthetic reactions. It is interesting to note that under the conditions of the experiment, degradation apparently proceeded almost exclusively through the oxidative deamination of glutamate to α-ketoglutarate rather than by decarboxylation to γ-aminobutyrate. Different plants apparently have widely varying capacities for the decarboxylation of glutamate as the studies of Schales et al (8) have already indicated. The data of table II also show, however, that significant amounts of glutamate are used for the formation of peptide bonds both in glutathione and in protein. This incorporation of glutamate into protein has been found to increase steadily with time at the expense of the free glutamic acid already accumulated by the cells.

Table II

Partition of Radioactivity of Glutamic Acid into Protein and Non-protein Fractions of Bean Hypocotyl Sections *

Fraction	Radioactivity as % of total radioactivity absorbed by cell
Protein	15.5
Cellular debris	4.2
Non-protein	80.3
Glutamic acid	43.0
Glutamine	18.1
Glutathione	1.3
α-Ketoglutaric acid	4.6
γ-Aminobutyric acid	0.9
α-Aminobutyric acid	0.0
Unaccounted for	12.4

* Sections were incubated for 1 hr at 25° C with 0.01 M potassium L-glutamate containing 10 μc of radioactivity. The total C^{14} assimilated gave 29,300 cpm; this is about 0.1 % of the C^{14} added as glutamic acid.

Incorporation by Different Plants: Table III illustrates that each of six different species of plants incorporated significant amounts of C^{14} from glutamate into their cellular protein. That this radioactivity is incorporated in the form of glutamate was established by hydrolysis of the protein and separation of the liberated amino acids by chromatography. Glutamate alone showed significant radioactivity as determined by radioautography (2).

The amino acid bound to protein by the process reported on here is undoubtedly bound through a peptide bond. Results of the treatments indicated

Table III

Incorporation of Radioactive Glutamate into the Proteins of Various Plant Tissues

Plant and tissue	Glutamate incorporated into total protein (μM/gm protein × hr)
Bean hypocotyl sections	0.35
Carrot root slices	0.38
Potato tuber slices	0.16
Pea seedlings	0.27
Oat seedlings	0.23
Young spinach leaves	0.07

in table IV make it seem highly unlikely that the amino acid is merely adsorbed on the protein. The failure of boiling trichloroacetic acid or of dialysis against alkali to remove significant activity argues against a physical adsorption, while the stability of the amino acid to performate treatment indicates a lack of binding to the protein through sulfhydryl groups. Likewise, the failure of ninhydrin to release radioactive CO_2 from uniformly labeled amino acid indicates a linkage involving either the carboxyl or amino group of the amino acid. Even the partial destruction of the protein with trypsin failed to release any great quantity of amino acid. In addition, the dependence of incorporation by mitochondrial protein on energy sources (ATP, Krebs cycle intermediates) and its inhibition by respiratory inhibitors (12) does not argue in favor of adsorption.

INCORPORATION INTO VARIOUS CELLULAR FRACTIONS: When intact tissue is allowed to accumulate radioactive glutamate and is then fractionated into protein fractions composed of the various cellular components, it is found that the amount of incorporated radioactivity is quite different in these different cellular fractions (table V). Most noteworthy is the relatively large incorporation into the microsomal or small particle fraction. The results indicate that the most active turnover of amino acids in the cellular proteins is associated with this fraction. The fraction designated arbitrarily here as microsomal actually encompasses particles that sediment in a number of distinct groups between 10,000 and $70{,}000 \times g$ (6). Thus, it is possible that only one type of this presumably heterogeneous set of particles is so active in incorporation, but such a conclusion is dependent on further knowledge concerning the nature of the various cellular particles occurring in plants.

DISCUSSION

Several noteworthy results have been obtained during the present investigation. First, the experiments show that the accumulation of at least some amino acids by plant cells is dependent on respiratory energy and is thus possibly similar to the process of active accumulation of other substances. In addition they establish that different cellular proteins possess different capacities for incorporation, thus giving some indication of the possible sites of most active protein turnover in plant cells. In this connection, a further fact is of particular interest. The nucleus has often been suggested as the center of protein synthesis in the cell (5). If there is in fact a direct relation between nuclei and protein synthesis then it would be expected that nuclei would show marked capacities for incorporation of amino acids into protein as compared with other cellular entities. Actually, however, nuclei exhibit just the opposite behavior and have consistently shown a smaller amount of incorporation than the other cellular bodies. In contrast, the microsomes exhibit markedly great amounts of incorporation. These findings, though not definitive because they are confined to observations at one time period only, nevertheless raise a question as to the relative roles of the various cellular entities in protein synthesis. It would appear that much more information (including rate studies on incorporation by cellular fractions) is needed before any one cellular fraction can be assigned an unequivocal role in protein synthesis.

TABLE IV

STABILITY OF THE INCORPORATED AMINO ACIDS TO VARIOUS TREATMENTS OF THE ISOLATED RADIOACTIVE PROTEIN

TREATMENT	AMINO ACID ADDED *	
	GLUTAMIC ACID-C^{14}	GLYCINE-C^{14}
None	100	100
Boiled with 5% trichloroacetic acid	98	99
Dialysis against dilute alkali	100	98
Radioactivity released by ninhydrin	3	2
Dispersion in performic acid	100	100
Partial hydrolysis with trypsin	95	96

* Values given represent specific activity as a percent of the specific activity of the original protein, isolated and washed as described previously.

TABLE V

DISTRIBUTION OF THE INCORPORATED C^{14} FROM GLUTAMATE AMONG CELLULAR FRACTIONS OF BEAN HYPOCOTYL SECTIONS

FRACTION	GLUTAMATE INCORPORATED (μM PER HR PER GM PROTEIN)
Homogenate	0.36
Nuclear	0.15
Mitochondrial	0.43
Microsomal	1.18
Soluble	0.31

SUMMARY

Radioactive glutamate is accumulated by plant cells from the external medium. This accumulation is inhibited by respiratory inhibitors and by dinitrophenol and thus appears to be linked with the production of respiratory energy. Glutamate is rapidly metabolized in the cell to glutamine and to α-ketoglutarate but only slightly to γ-aminobutyrate. It is incorporated into the tripeptide glutathione and into cellular proteins. The incorporation into protein is a general reaction which occurs in a variety of plants and apparently involves the actual formation of peptide bonds. Different cellular fractions have widely different capacities for glutamate incorporation with the greatest incorporation during an hour occurring into the microsomal fraction, and the least into the nuclear fraction.

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THE EFFECT OF TEMPERATURE ON THE BEHAVIOR OF MALIC ACID AND STARCH IN LEAVES OF BRYOPHYLLUM CALYCINUM CULTURED IN DARKNESS[1, 2]

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It has been known for many years that the temperature at which excised leaves of *Bryophyllum calycinum* are cultured in darkness has a considerable effect upon the extent to which the tissues become enriched in organic acids. In 1938, Wolf (14) presented data to show that, at a temperature of 7 to 8° C, the production of acid and the utilization of starch were apparently both stimulated as compared to the behavior of these substances in leaves cultured at about 22° C, and similar observations have been reported in a previous paper from this laboratory (4). Additional evidence with respect to the favorable effect of low temperature on the formation of acid has recently been given by Thomas and Ranson (5).

In the earlier work from this laboratory, however, the observations were made only after culture for 23, 66, and 142 hours at temperatures of 20°, 9°, and 1° C. Furthermore, the sampling method used at that time for collecting the leaflets was far less satisfactory than that presently employed. It has seemed desirable, therefore, to reinvestigate the matter with more detailed attention to the early phases of the chemical transformations that occur and with the use of improved sampling methods. A better opportunity has thus been afforded for the consideration of the chemical relationships between the quantities of starch and malic acid involved in the reactions, and for the study of the somewhat subtle alterations which take place in the general course of the chemical events when the period of culture under artificial conditions exceeds the normal length of a night.

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Experimental Procedure

Plantlets of *Bryophyllum calycinum* from the clone that has been maintained in this laboratory for a number of years were transplanted to sand in pots in the greenhouse March 27, 1951, and were grown with use of a culture solution. By July 9, the plants bore several pairs of well-developed 3-leaflet leaves. On this date, 12 samples were collected by the statistical method (11) from 36 plants, using the 12 leaflets from the two uppermost pairs of fully-expanded leaves. Each sample thus contained 36 leaflets, one of which was derived from each plant, and each of the 12 leaflet positions was represented three times in each sample. Collection was started at 3 P.M., the weather being clear and sunny as it had been the previous day.

The samples were at once weighed to five significant figures and sample 1 was placed in the drying oven at 4:00 P.M., this being taken as the zero hour of the experiment. Meanwhile, the leaves of the other samples were arranged along the sides of V-shaped troughs, samples 2 to 6 in a room maintained at 24° C and 75 % relative humidity, samples 7 to 12 in a room at 6° C, and enough water was added to immerse the petioles. The humidity in the cold room was uncontrolled but was fairly low. The rooms were darkened at 4:00 P.M., and the conditions were maintained for 40 hours at 24° C and for 48 hours at 6° C. Successive samples were removed at

the times shown in table I, weighed, and dried at 80° C. After being dried, the leaflets were broken up and exposed to a temperature of 24° C and 75 % relative humidity until they had assumed constant weight (roughly 2 weeks), being finally weighed to four significant figures. The samples were then ground in a Wiley mill in the same room and bottled. The bottles were stored in darkness in the air-conditioned room and were removed only when taken to the balance to weigh out aliquots for analysis. Experience has shown that attention to these details of technique greatly improves the over-all accuracy of the work.

The weights of the individual samples, together with evidence on the constancy of their fundamental composition, are shown in table I. Probably because of the high temperature of the greenhouse at the time of collection, the leaves were not fully turgid and crisp when picked but they soon became so when placed in water, and it will be noted that the samples promptly increased in fresh weight during treatment, the mean increase per sample being 7.4 gm. Presumably because of the deficient water content, the coefficient of variation of the fresh weight of this set of samples was somewhat larger than is usually observed with samples of Bryophyllum leaves collected by the statistical method, but that the chemical composition was satisfactorily constant is shown by the low coefficient of variation for total N and ash. The protein N also remained essentially unchanged.

The equilibrated dry weight per kilo of initial fresh weight is used in computing the composition of the samples in gm per kg of initial fresh weight. This quantity, when multiplied by the result of an analytical determination expressed as a percentage and divided by 100, yields the composition in the units desired. It is in order that the factor for each sample shall be known to four significant figures that the fresh weights and equilibrated dry weights are obtained with the accuracy mentioned.

The analytical methods currently in use in this laboratory have recently been described in detail (13).

Experimental Results

Figure 1 shows the organic solids and carbohydrates in the series of samples, the data for those cultured at 24° C plotted as solid lines and for those at 6° C as broken lines. For the determination of organic solids, analytical samples are dried at 110° C to give the total solids and are then ashed in a muffle at 600° C. The weight of the ash is deducted from the total solids, the difference being taken as the organic solids. However, the ash so obtained contains a substantial proportion of carbonate which is derived from the combustion of the organic acids. Strictly speaking, therefore, the carbonate is organic in origin and should be reckoned not as a part of the ash but should be added to the organic solids. This correction has not been applied in the present case since the alkalinity of the ash was constant for all samples and, accordingly, the correction for carbonate is also constant. If the correction had been applied, the curves for organic solids in figure 1 would merely be placed approximately 7 gm per kg higher on the diagram; the shape of the curves would be unchanged.

The organic solids of the leaves cultured at 24° C increased by 2 gm during the first 16 hours in darkness but subsequently slowly diminished. At 6° C, the initial increase was almost the same but was more rapid, and the increased weight was maintained

Table I

Fundamental Data on Samples of Bryophyllum calycinum Leaves Collected by the Statistical Method and Subjected to Culture in Water in Darkness at 24° C (Samples 2 to 6) and at 6° C (Samples 7 to 12)

Sample no.	Culture period	Per sample			Per kilo initial fresh weight			
		Fresh weight at start	Fresh weight at end	Equilibrated dry weight	Equilibrated dry weight	Total N	Protein N	Ash
	hr	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
1	0	145.01	145.0	18.40	126.9	2.18	1.87	13.1
2	8	152.74	158.1	19.27	126.2	2.17	1.80	13.2
3	16	144.72	151.4	18.54	128.1	2.19	1.78	13.3
4	24	152.84	160.5	19.33	126.5	2.23	1.77	13.2
5	32	146.67	154.0	18.29	124.7	2.24	1.81	13.6
6	40	154.33	161.6	19.20	124.4	2.18	1.75	13.1
7	8	149.66	153.5	19.23	128.5	2.18	1.81	13.4
8	16	146.23	152.1	18.53	126.7	2.18	1.86	13.1
9	24	150.65	157.2	19.33	128.3	2.19	1.80	13.5
10	32	156.33	164.5	20.29	129.8	2.18	1.80	13.6
11	40	153.12	161.8	19.57	127.7	2.18	1.83	13.3
12	48	150.53	158.4	19.57	130.0	2.21	1.83	13.1
Mean		150.24	157.6	....	....	2.19	1.81	13.3
S.D.		± 3.8	± 4.4	....	....	± 0.02	± 0.035	± 0.19
C.V. (%)		2.6	2.8	....	....	1.0	1.9	1.4

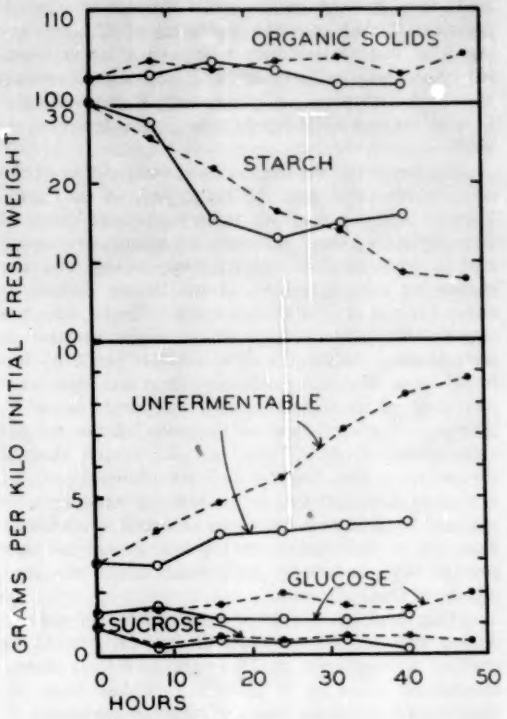

FIG. 1. Changes in organic solids and carbohydrates of excised leaves of *Bryophyllum calycinum* cultured in water in darkness at 24° C (solid lines) and at 6° C (broken lines).

with minor fluctuations for much longer. In general, it would seem that the effect of culture at low temperature consists mainly in postponing the time at which loss of organic solids becomes apparent (4).

All of these changes in weight were, however, small and at the limit of accurate measurement, if the sampling and analytical errors are taken into consideration. The data contrast with results obtained with leaves picked at sunrise at the time of high acidity and low starch content (9), for, with such material, significant loss of organic solids soon becomes established on culture in darkness. However, it is to be noted that leaves picked in the afternoon and cultured in darkness for 16 hours are physiologically at about the same stage in the course of their general daily metabolism as leaves picked at sunrise. Thus the slow loss of organic solids that began in the present leaves after the expiration of 16 hours in darkness at 24° C may probably be regarded as analogous to the loss observed in samples picked at sunrise and kept in the dark.

The behavior of the starch is also shown in figure 1. The leaves contained a little more than 30 gm per kg when collected, but half of this had disappeared after 16 hours at 24° C. There was a small additional loss during the next 8 hours but further culture in the dark led to a small but significant *increase* in starch. This phenomenon, synthesis of starch in Bryophyllum leaves in darkness, is one that has been encountered several times in experiments carried out in this laboratory (9) and has been found to be correlated in point of time with a loss of malic acid.

At 6° C, starch diminished throughout the period of the experiment at a rate which for 40 hours can be expressed as an approximately straight line. Although the leaves still contained a little more than 16 gm of starch after 40 hours at 24° C, they retained only 8.6 gm after culture for the same period at 6° C. Starch diminished more rapidly in the first 16 hours at the higher temperature than at the lower, but a stage was then reached at which reactions that ultimately led to the formation of a small quantity of starch gradually assumed the ascendancy. At no time within the first 40 hours at the lower temperature were such reactions apparently able to influence the rate of decomposition of starch that was established in the first 16 hours. Only in the last 8 hours of the experiment did the rate of disappearance of starch diminish. Interpretation of this result can be at best speculative, but there is a possibility that the reactions which at the higher temperature finally led to the formation of a small amount of starch were suspended or slowed down to negligible proportions at the lower temperature. Such a view would account for the apparent promotion of the utilization of starch in Bryophyllum leaves cultured at low temperatures.

The effects of culture on the simple carbohydrates of the leaves are shown at the bottom of figure 1 plotted on a scale four times as large as that used for starch. It is clear that neither glucose nor sucrose were influenced to any marked extent and that both substances were present in only minor proportions. The component recorded as unfermentable carbohydrate was, however, unusually high in this set of samples, and reactions occurred which led to a considerable increase at the lower temperature. Starting at a level of 3 gm per kg, no less than 8.8 gm were present after 48 hours at 6° C. In sharp contrast, only 4.2 gm were present after 40 hours at 24° C. Culture at low temperature would therefore seem to have stimulated reactions that led to the formation of a carbohydrate material which is not attacked by yeast.

One of the more important unfermentable carbohydrates known to be present in Bryophyllum leaves is sedoheptulose and determinations of heptose were accordingly made by the method of Dische (1). The control sample before culture was found to contain only 0.25 gm per kg, a quantity which is less than 10 % of the total amount of unfermentable carbohydrate present. At 24° C, this increased progressively to 0.8 gm per kg but, at 6° C, heptose *diminished* to 0.1 gm. Clearly, therefore, the unfermentable carbohydrate which increased so spectacularly during culture at low temperature was not sedoheptulose. This observation is the more interesting since

a heptose, presumably sedoheptulose, is synthesized in appreciable quantities in the later stages of culture of Bryophyllum leaves at 21° to 26° C in continuous light (10).

The behavior of the organic acids of the leaves is shown in figure 2. For the first 8 hours, owing to the formation of acids, the pH dropped at the same rate at both temperatures. At 24° C, this fairly rapid rate was maintained for another 8 hours, but there was little further change and a slight increase in pH occurred later. On the other hand, at 6° C, the rate of decrease of pH diminished sharply in the interval between the observations at 8 and at 24 hours and the subsequent change in pH was slow. Nevertheless, the pH continued to drop throughout the period of the experiment.

The curves for the change in pH are accurately reflected in form by the curves that show the change in the total organic acidity. Organic acids were synthesized rapidly in the first 16 hours at 24° C but subsequently diminished slightly. At 6° C, organic acids were formed continuously throughout the experiment so that after the expiration of 32 hours, the leaves at low temperature contained a larger quantity than those cultured at 24° C and the differential increased with time. A promotion of the accumulation of organic acids by culture at low temperature is thus clearly evident.

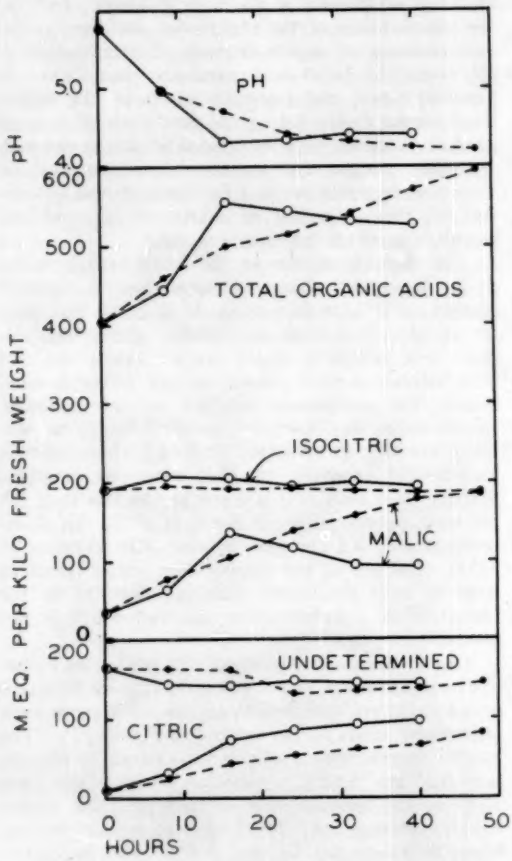

FIG. 2. Changes in organic acid composition and pH of excised leaves of *Bryophyllum calycinum* cultured in water in darkness at 24° C (solid lines) and at 6° C (broken lines).

The details of the organic acid composition of the samples are plotted in the lower part of the figure. Isocitric acid is the major organic acid component of Bryophyllum leaves collected on sunny afternoons and it appears that the quantity present did not change to any significant extent during culture at either normal or low temperatures. To be sure, the curves suggest that there was a slight increase in isocitric acid during the first 8 hours at 24° C followed by a slow and continuous drop and that similar although smaller changes occurred at 6° C. However, the coefficient of variation of the six determinations at 24° C was only 3.2 % and that of the seven values (initial and six observations) at 6° C was only 1.6 %. It is doubtful that isocitric acid can be determined with an accuracy much better than 3 % so that there is no convincing evidence that isocitric acid underwent real change under the conditions of this experiment.

Malic acid, on the other hand, increased rapidly during the early hours of culture. At 24° C, it reached a maximum in 16 hours and then slowly diminished following a pattern that has been repeatedly observed in long continued experiments in darkness both in this laboratory and elsewhere. The important point to note is that after culture of the leaves in darkness for a period roughly that of a night, a change in the metabolism of malic acid becomes apparent and, instead of accumulating, it begins to diminish.

The behavior at 6° C was quite different. For 24 hours, malic acid increased at a rate that can be fairly accurately expressed by a straight line and the rate of formation fell off very little during the next 16 hours. At the 24 hour point, more malic acid had been formed in these leaves than in those at 24° C and the surplus increased considerably as the period of culture was prolonged.

Citric acid is low in Bryophyllum leaves picked in the afternoon and is known to undergo diurnal variation under normal conditions in a manner parallel to although less extensive than malic acid (8). It is also known to increase for many hours in leaves cultured in darkness whether these were picked in the afternoon, or at sunrise when citric acid is present in appreciable proportions (9). In the present experiment, citric acid likewise increased, rapidly at first and then more slowly; however, the curve for the observations at 24° C lies well above that for 6° C, and even after 48 hours at 6° C there was less citric acid present than after 24 hours at 24° C. Accordingly, the behavior of citric acid is also dependent upon the temperature, but the significant point is that the effect for a period of at least 40 hours is the converse of the effect upon malic acid.

In the early stages of culture, the formation of citric acid is apparently stimulated at the *higher* temperature.

However, an earlier experiment carried out in this laboratory (4) showed that, if culture is greatly prolonged, the quantity of citric acid formed at 9° C ultimately exceeded that formed at 20° C. The data suggested that, after 66 hours at 20° C, reactions which led to the utilization of citric acid gradually assumed dominance, although little evidence of such reactions was noted in the experiment at 9° C. Accordingly the general behavior of citric acid is fundamentally identical with that of malic acid; the differences observed merely represent differences in the rates of the reactions which affect malic acid on the one hand and citric acid on the other and in the effect of temperature upon these rates.

Data for the so-called undetermined acids of the leaves are also plotted in figure 2. Although the intrinsic accuracy is not high since the results are obtained by difference, it appears that there was a small decrease in the undetermined acids early in the culture period at 24° C but that a similar decrease was delayed in the leaves cultured at 6° C until nearly 24 hours had elapsed. The changes in this analytical component were, however, small and impossible to interpret in the absence of knowledge of the identity of the substances concerned.

Discussion

Consideration of the data that have been presented suggests that the main effect of culture of Bryophyllum leaves at low temperature is to depress the rates at which certain of the metabolic reactions occur. The leaf components chiefly concerned are starch on the one hand and malic and citric acids on the other. The quantity of each of these substances present under any defined set of conditions is the resultant of enzymatically controlled reactions, one or more of which in each case leads to the synthesis of the substance and one or more to its decomposition or utilization. In such experiments as these, the temporary concentration relationships among the reactants at each of several stages is observed, and the progressive changes in the quantities of the substances present furnish evidence of the effect of the experimental treatment upon the rates of the reactions in which these substances are concerned.

A clearly defined example is provided by the surprising behavior of the unfermentable carbohydrate. This material, whatever its chemical nature may be, is obviously being synthesized as the culture period progresses. However, its slow accumulation at 24° C suggests that it is also being utilized for the formation of some further product as yet unknown. At 6° C, the rate of these latter reactions is so diminished that a substantial accumulation of unfermentable carbohydrate occurs. In physico-chemical terms, the temperature coefficient of the net reactions which lead to synthesis is quite different from that of the reactions which lead to utilization.

With respect to the main effect under study, the apparent stimulation of the formation of malic acid at low temperature, the data indicate that synthetic reactions proceeded at a fairly high net rate at both temperatures at the start but continued at this high rate for a somewhat longer time at 24° C than at 6° C. After about 16 hours at 24° C, however, reactions that led to the loss of malic acid supervened and soon dominated the situation whereas, at 6° C, the effect of such reactions is scarcely detectable. The analogy with the behavior of the unfermentable carbohydrate is thus complete.

If it may be assumed that the metabolic reactions observed in the present lot of leaves were fundamentally the same as those seen in previous sets of samples studied in this laboratory (4, 9), citric acid behaved in a similar manner although with this substance the reactions that led to synthesis were slower than those by which malic acid was produced. Furthermore, the reverse reactions which lead to the loss of citric acid do not become effective until after the lapse of many hours. The curve for the citric acid content of the leaves in the earlier experiment leveled off or turned downward only after about 90 hours of culture at room temperature in the dark. No experiment has yet been carried out for a sufficient length of time at low temperatures to demonstrate an ultimate loss of citric acid, and the data at hand suggest that a period far longer than 140 hours would be required. Nevertheless, the maximum which citric acid may reach (4) was greater at low than at high temperature and the general behavior was thus closely analogous to that of malic acid, only the time scale is different.

The behavior of starch in this as well as in earlier experiments was, with surprising accuracy, the converse of that of malic acid. The respective curves in figures 1 and 2 are essentially mirror images of each other and the correlation coefficients calculated from the data support this impression. At 24° C, the correlation coefficient between malic acid and starch was -0.89 ($r = 0.882$ for $P = 0.02$ with 4 degrees of freedom). At 6° C the correlation was even better, the coefficient being -0.99 ($r = 0.951$ for $P = 0.001$ with 5 degrees of freedom). Although the maximum does not correspond exactly in point of time with the minimum value for starch, the general behavior is exactly that to be anticipated if interconversion of these two substances were assumed to be taking place. The statistical analysis suggests that in the experiment at 24° C there is less than one chance in 50 that the apparent relationship is fortuitous and at 6° C, as well as in several other cases (8, 10), far higher correlations have been found.

In view of these obvious relationships, calculations were made of the relative molar quantities involved in the transformations. If malic acid arises from a sequence of enzymatic reactions in which starch is the starting material, there should be a constant ratio between the number of moles of acid produced and the number of moles of starch used. However, if there are competing reactions which draw upon malic acid, or possibly upon one or other

of its precursors, the ratio will change as the culture period continues. Figure 3 shows a plot of the ratios between the number of moles of malic acid that have been formed and the number of moles of starch ($C_6H_{10}O_5$ being taken as 1 mole) that have disappeared at each point of observation. The data thus refer to the cumulative effect of all of the reactions that have occurred up to each point of observation. At the end of the first 8 hours at 24° C, for each mole of starch that had disappeared, 0.93 moles of malic acid had been synthesized but, as time went on, the ratio dropped steeply so that after 16 hours it had diminished to 0.56. In the first 8 hours, the quantities involved were small amounting to only 15 millimoles of starch and 14 millimoles of malic acid. It was in the next 8 hour period that the major reaction occurred, for no less than 77 millimoles of starch vanished and 37 millimoles of malic acid were formed. The ratio for the interval between 8 and

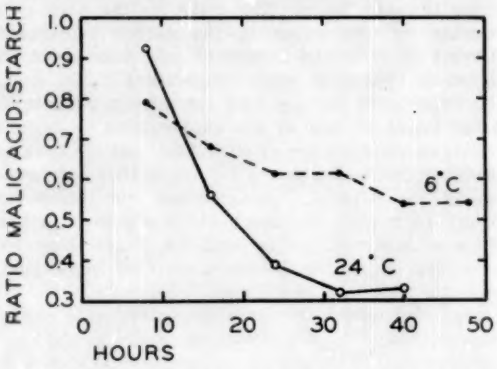

FIG. 3. Molar ratio of the cumulative change in malic acid to the cumulative change in the starch content of excised leaves of *Bryophyllum calycinum* cultured in water in darkness at 24° C (solid line) and at 6° C (broken line).

16 hours was 0.49; that is to say, on the average one-half mole of malic acid appeared for each mole of starch used.

The subsequent behavior of the starch and malic acid at 24° C (figs 1, 2) reflects the increasing dominance of reactions which represent a change in the course of the metabolism. Only a little more starch was used and it finally began to increase; in addition, malic acid began to disappear slowly. The actual quantities of substance involved were small but the curve for the ratio (fig 3) flattens and becomes constant at about 0.33. The alteration in the course of the metabolism may possibly be associated with the increasing physiological stress as the period of darkness began to exceed that of a normal night but is clearly not to be attributed to a deficiency of starch. At the expiration of 16 hours at 24° C, only half of the starch initially present had disappeared.

The curve for the molar ratio between malic acid and starch at 6° C (fig 3, broken line) differs considerably from that at 24° C. During the first 8 hours, approximately 0.8 moles of malic acid were formed per mole of starch decomposed and the ratio which shows the cumulative effect diminished rather slowly over the experimental period to 0.54. As is evident from figures 1 and 2, the loss of starch and gain of malic acid proceeded at a fairly constant rate throughout. Nevertheless, the continuous although slow drop of the ratio is in conformity with the view that what may be referred to as the efficiency of the transformation was gradually and increasingly being interfered with by reactions which led to the utilization of malic acid or of a precursor of this substance. At 6° C, however, such reactions were unable at any time within the experimental period to dominate the situation as they so promptly did at 24° C.

The evidence thus suggests that malic acid is, as it were, a centrally located substance in a complex series of metabolic reactions, some of which lead to its increase, some to its diminution. Starch would seem to play the part of a storage substance which, in darkness, rapidly begins to contribute to the reactions that synthesize malic acid. The molar ratios are such in the present case that all of the C of the newly formed malic acid may have arisen from starch. However, this is by no means always so, for, in an experiment described in a previous paper (8), the molar ratios of malic acid to starch during two successive 9 hour periods of darkness, the leaves having been exposed to light in the intervening 16 hours, were respectively 1.46 and 1.96. Ratios as high as or even higher than these have been observed in other experiments, and such observations clearly demonstrate that not all of the C of the newly formed malic acid could have been derived from the starch. Convincing proof of this is to be seen in the experiments of Thurlow and Bonner (6), Varner and Burrell (7), and, recently, of Thomas and Ranson (5) in which leaves have been exposed in darkness to an atmosphere containing radioactive CO_2. The malic acid promptly became labeled with radioactive C.

Synthesis of citric acid occurred in the present set of samples to an extent that is only a little less striking than the synthesis of malic acid. At the end of 8 hours at 24° C, 6.9 millimoles of citric acid had been formed as compared with 14 millimoles of malic acid. After 16 hours, the figures were, respectively, 20 and 51.6. Thus, for every mole of citric acid formed, from 2 to 2.5 moles of malic acid also made their appearance. Subsequently, owing to the slow loss of malic acid and the continued synthesis of citric acid, the ratio between the total quantities formed decreased so that after 40 hours the increase in citric acid equalled that of malic acid, both being close to 29 millimoles per kg of leaf tissue.

At 6° C, the rate of synthesis of citric acid was much depressed although synthesis of malic acid continued at a fairly rapid rate. Accordingly, the ratio between the molar quantity of malic acid formed to that of citric acid was higher. After 8 hours, it was

4.5 and after 16 hours 3.3; subsequently it remained essentially constant at about 3.7. Thus, at 6° C, nearly 4 moles of malic acid were synthesized for each mole of citric acid that appeared, and this ratio was approximately maintained throughout the period of study.

That the formation of citric acid in Bryophyllum leaves is in some direct or indirect way related to the loss of starch is implicit in previous observations on the diurnal variation in acidity in response to exposure of the leaves to light and darkness, an especially clear case having been described in an earlier paper (8). The present data for citric acid and starch are also closely correlated, the coefficients of correlation being −0.95 at 24° C ($r = 0.917$ for $P = 0.01$ with 4 degrees of freedom) and −0.98 at 6° C ($r = 0.951$ for $P = 0.001$ with 5 degrees of freedom). Unfortunately, such calculations furnish no clue as to the chemical mechanisms which underlie the relationship. However, it is clear that citric acid played a relatively more important role in the present set of samples than is usually noted. This was especially marked in the leaves cultured at 24° C.

The failure of isocitric acid to become involved to any significant extent in these complex reactions is in conformity with most of the previous observations obtained by adequate analytical methods in this laboratory. In work carried out before the aconitase method of Krebs and Eggleston (3) had been applied by Hargreaves, Abrahams and Vickery (2), it had been necessary to calculate the content of isocitric acid by difference, a procedure that frequently led to a false impression of the reactivity of this substance. In view of this, no comment is justified upon the apparent failure of the so-called undetermined acid fraction to undergo striking change. All that can be said is that the undetermined acids of this set of samples were not as a group notably affected by culture either at normal or at low temperature. Individual components may well, however, have been extensively involved. The development of both qualitative and quantitative information regarding these substances is urgently needed.

One final point may perhaps deserve comment. As experience with the behavior of the chemical components of Bryophyllum leaves is extended, it has become increasingly clear that different sets of samples, although satisfactorily constant in composition within the set, may differ quite widely in composition from each other. The general behavior of the components appears to follow fairly well defined pathways which it is the purpose of these investigations to study. Nevertheless, the details of the reactions, when subjected to somewhat penetrating analysis, are often found to differ from one set of samples to another. This is particularly true of such matters as the molar relationships between the several substances concerned in the main metabolic reactions. Accordingly, all general statements must be qualified, for they are true only within what are unfortunately still somewhat broad limits.

Summary

Leaves of *Bryophyllum calycinum* picked in the afternoon at the time of high starch content have been cultured in water in darkness at 24° C and at 6° C in order to study the early phases of the effect of temperature on the rate and extent of the formation of organic acids and on the utilization of starch. About one-half of the starch present disappeared within 16 hours at 24° C but subsequently, after a small further loss, it increased slightly. Malic acid increased rapidly for 16 hours but then slowly decreased, thus behaving in a manner closely but inversely correlated with that of starch. This is strong evidence that starch contributed to the synthesis of malic acid. Citric acid increased continuously during the 40 hour period of the experiment, although at a rate considerably slower than malic acid.

At 6° C, the rate of disappearance of starch was somewhat less than at 24° C but remained substantially constant throughout so that after 40 hours nearly 75 % of the starch had been used. The rate of formation of malic acid was a little slower than at 24° C but was maintained nearly constant over the entire period of study. Thus, after 40 hours the leaves contained nearly twice as much malic acid as those cultured for the same time at 24° C. The apparent stimulatory effect of low temperature on the accumulation of this substance is thus amply confirmed. The rate at which citric acid accumulated was much slower than at 24° C but was continuous for 48 hours.

The observed effects are tentatively ascribed to a difference in the influence of temperature on the rates of the reactions which on the one hand lead to the synthesis of malic acid and the utilization of starch and on the other hand to the loss of malic acid. The net rate of these latter reactions appears to be depressed at low temperature so that malic acid accumulates continuously whereas at higher temperatures it reaches a maximum and then diminishes. Similar considerations seem to apply to the behavior of an unfermentable carbohydrate component which is not sedoheptulose. They also appear to be applicable to the behavior of citric acid, but with this substance the effects are only slowly established and would require a prolonged period of culture for their full demonstration at low temperature.

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BRIEF PAPERS

SMOG INJURY AND RUST INFECTION[1]

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The accidental observation that the rust-infected portions of bean leaves showed no injury when non-rusted leaves or portions of leaves showed severe damage from natural smog in the greenhouses in Berkeley, suggested an exploration of the phenomenon under controlled conditions.

Beans (*Phaseolus vulgaris* L. var. Pinto) or sunflowers (*Helianthus annuus* L. var. Mammoth Russian) grown in 4- or 5-inch pots in the greenhouse were used at 10 to 18 days from seeding. In most trials plants grown and inoculated in Berkeley were transported to Riverside and treated in the fumigation chambers there. Primary leaves were inoculated with rust by dipping a brush in a suspension of uredospores (*Uromyces phaseoli* (Pers.) Wint. or *Puccinia helianthi* Schw.) and applying the brush to one half of the lower leaf surface. Infection was usually apparent in 4 days and the pustules opened in 9 days. Smog injury (4) was rated on a scale of 0 to 10 in which 0 indicated no silvering and bronzing of the lower leaf surface and 10 indicated that silvering and bronzing extended over the entire leaf surface. The degree of discoloration was excellently correlated with the degree of later injury and with the later death of severely injured plants.

The two glass fumigation chambers received water-scrubbed, carbon-filtered air from a single source. The cleansed air was delivered at the rate of about 1 change every 4 minutes. The phytotoxicants responsible for smog damage (1) were derived by combining gasoline vapors with ozone in a glass reaction tube inserted into one of the two chambers. Plants placed in the fumigatorium not receiving the ozonated olefins served as controls. The degree of injury has been found (3) to be directly proportional to the length of fumigation at a given concentration. The concentration of ozonated olefins in these experi-

[1] Received November 7, 1953.

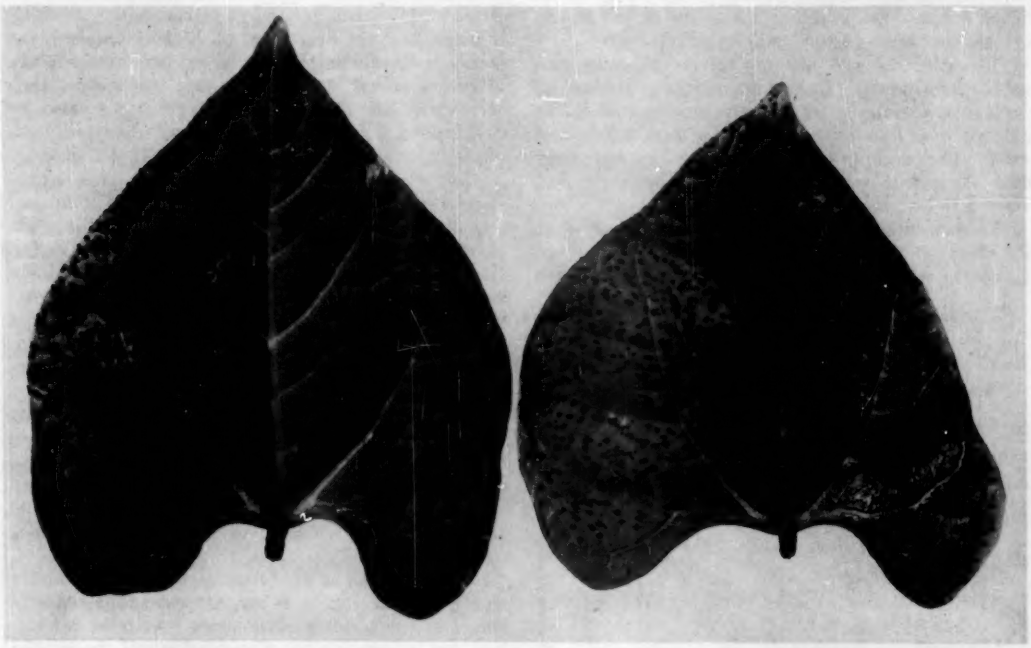

FIG. 1. Smog injury on primary leaves of Pinto bean. The left side of each leaf was inoculated with rust on January 29, 1953. The left leaf was not further treated. The right leaf is from a plant treated with artificial smog for 8 hours on February 6. Photographed February 8, natural size. Smog damage is apparent principally on the right half of the right leaf, but has extended to the left half at places well distant from the rust. As the rust mycelium extends only about 0.5 mm beyond the visible rust, it is clear that for some distance beyond the mycelium the rust infection has exerted a protective action against smog.

ments ranged from 0.4 to 0.5 ppm, based on the release of iodine from potassium iodide in an ozone-free atmosphere.

Bean plants of which half leaves had been infected with rust for 3, 5, 7, or 9 days were exposed to artificial smog of 0, 3, 6, 9, 12, or 24 hours. The next day they were rated for smog symptoms and smog damage. Results given in figures 1 and 2 show that leaves infected with rust consistently exhibited less smog damage than non-rusted portions of the same leaves. The greatest reduction in smog injury resulted from 7-day rust infection. It can be estimated by interpolation that it required about 5 times as long an exposure to smog to cause the same damage on leaves infected for 7 days with rust as was required on non-rusted leaves.

Two-day rust infection caused no apparent reduction in smog damage in one trial; 3-day rust infection reduced smog damage in two trials. It appears that the protective action of rust infection against smog damage becomes apparent at about 3 days after infection and increases with age of infection up to about 7 days, but there are some inconsistencies in the data.

In two trials sunflowers partly infected with rust for 7 and 9 days before smog treatment for 8 hours were protected from smog damage in the rusted areas. When the rust was killed with heat the smog protecting nature of the rusted tissues was retained.

The effect of rust was not merely to delay ozonated olefin injury. Rusted leaves which showed no damage at one day after exposure to ozonated olefins did not show ozonated olefin injury in a subsequent week. The possibility that rust-infected plants might show the growth suppression described by Koritz and Went (2) was not investigated.

To determine if the living rust was necessary for the observed rust effect, half-rusted bean leaves were dipped in water at 45° C for 90 seconds to kill the rust without apparent host injury (7). Plants on which one leaf was heat treated 3, 5, 7, 9, 10, or 14 days after inoculation, and in which the interval from heat treatment to ozonated olefin treatment was 1 hour, 2 days, or 6 days, all showed freedom from ozonated olefin injury of the tissue in which the rust was killed.

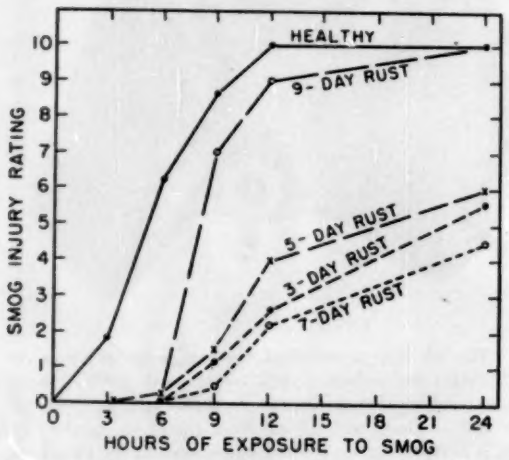

FIG. 2. Quantitative relation of duration of smog exposure and age of rust infection to smog injury.

Heat treatment of healthy tissues reduced ozonated olefin injury significantly but the effect was short lived. In one trial when healthy tissue was heat treated 30 minutes before exposure to the phytotoxicant, no injury resulted, but where the heat treatment preceded the exposure to ozonated olefins by 24 hours no protection from ozonated olefin injury was found. The untreated, opposite leaves on plants of which one leaf was dipped in water at 45° C for 90 seconds and the entire plant exposed to ozonated olefins showed less injury than leaves of untreated plants, but more than that of the opposite heat-treated leaf. Whether this protection of the leaf, which was not intentionally treated, was due to a transmitted effect from the opposite, treated leaf or was due to an independent effect of heat owing to inadequate insulation of the untreated leaf, was not determined. Treatment in water at 22° C before exposure to artificial smog had no apparent effect. It is concluded that the effect of immersion of leaves in water at 45° C is a heat effect rather than a water effect and that the heat effect on healthy tissues is too transient to explain the lack of ozonated olefin injury in rusted tissues in which the rust was killed. It is also concluded that the smog protection caused by rust infection is a residual effect not necessarily associated with the living rust.

The stomata near rust pustules on bean leaves show smaller openings during the day than do normal stomata. This might be thought to reduce absorption of smog and to explain the reduced smog damage. However, there is no direct evidence of reduced absorption of smog by rusted tissue, and there is actually greater absorption of sulfur vapors by rusted than by normal leaf tissue (8). Also some smog injury occurs with exposure of plants to smog in darkness, when the stomata are normally closed. Diseased tissues are generally more permeable than healthy (5, 6) and on this basis greater absorption of smog by rusted than by healthy tissue might be expected. If the latter is true, the explanation of the reduced smog damage must be sought in some mechanism in the rusted tissue which reduces the toxicity of smog to the leaf.

The extension of the smog protection beyond the rust mycelium (fig 1) is an indication that the substance or effect which causes smog protection can diffuse out a considerable distance beyond the source.

The high pantothenic acid content of rust-infected bean leaves (9) suggested that pantothenic acid might be responsible for lower smog damage of rusted tissue. Water solutions of 0.01 to 1 % calcium pantothenate were applied to beans in the transpiration stream, as a surface spray on the leaves, as an infiltrating spray, by vacuum infiltration, and as a sub-

strate for detached leaves. Some smog protection resulted from these treatments but the protection was not clearly greater than from treatments with water alone, and no specific effect of calcium pantothenate on smog damage has been clearly demonstrated. A great variety of plant treatments such as those described above may cause a reduction in smog damage, but a discussion of these is beyond the scope of this report.

Water extracts of rust infected tissues were not effective in reducing smog damage by the methods tested.

Summary

Bean and sunflower leaves infected with rust (*Uromyces phaseoli* or *Puccinia helianthi*) were less injured by natural or artificial smog than were otherwise similar, healthy leaves. To produce the same degree of smog, or ozonated olefin, damage on bean tissue which had been infected with rust for 7 days required about five times as long an exposure to smog as to produce the same injury in healthy tissues. Rusted tissue in which the rust mycelium was killed by heat was still resistant to smog damage. The protection from smog damage by rust infection could not be explained by stomatal closure or by the high content of pantothenic acid in the rust infected tissue, but appears to be due to some substance which diffuses beyond the limits of the rust mycelium.

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PRECURSOR RELATIONSHIPS IN LIGHT ACTIVATED ACCUMULATION OF NIACIN IN LEAF DISKS FROM NORMAL, LIGHT-STARVED, AND ZINC DEFICIENT TOMATO PLANTS[1,2]

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The role of tryptophan and 3-hydroxyanthranilic acid in the biosynthesis of niacin in heterotrophic organisms is well known (7, 8). Nason (9) has shown that tryptophan and 3-hydroxyanthranilic acid act as precursors in detached corn embryos, and Galston (3) has demonstrated a similar effect with etiolated pea epicotyl sections. Gustafson (4) was able to show a higher content of niacin in leaves of tomato, cabbage, and broccoli after incubation of the whole leaves with petioles immersed in tryptophan solutions in comparison with water controls.

This is a report on attempts to influence the previously reported (2) light activated accumulation of niacin in tomato leaf disks by infiltration of the leaf disks from normal, light-starved, and zinc deficient plants with the precursors in question.

The method of preparation of leaf disks from mature leaves, conditions of exposure to light, and evaluation of results have been described (2). For these experiments the disks were vacuum infiltrated with water or the appropriate solution. The process of infiltration was found to have no effect on the accumulation of niacin in these disks as compared with non-infiltrated disks. Niacin was determined by the microbiological method (*Lactobacillus arabinosus* 17-5) (1) after hydrolysis with 1 *N* sulfuric acid. Initial niacin content was determined on disks frozen immediately after infiltration; experimental disks were frozen after exposure.

DL-Tryptophan, L-tryptophan, and 3-hydroxyanthranilic acid did not increase the synthesis of niacin in normal disks in light, nor did they cause any synthesis in disks in the dark. At concentrations of over 5×10^{-3} M, DL-tryptophan invariably caused in-

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[2] Paper from the Department of Botany, University of Michigan, No. 1008.
[3] This paper constitutes a portion of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Michigan in 1953. The work was made possible by an F. C. and Susan Eastman Newcombe Fellowship in Plant Physiology. Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin.

hibition of niacin synthesis by 50 to 90 %. This inhibition was not observed when L-tryptophan was used. The conditions employed with each of these compounds were varied with respect to concentration (6×10^{-3} M to 5×10^{-6} M), pH (4.0 to 6.7), and incubation time (3 to 10 hours).

In a separate experiment, evidence that DL-tryptophan actually entered the cells was obtained by following changes in auxin content during incubation. Auxin content was determined on the ether extract of dried leaf disks according to the standard Avena curvature test as described by Went (13). As shown in table I tryptophan causes accumulation of auxin as previously reported by Wildman, Ferri, and J. Bonner (14) for spinach leaves and Tsui (12) for tomato. It is apparent that light does not affect this conversion. Similar effects were observed in two other experiments.

In view of the failure of the presumed niacin precursors to stimulate synthesis in normal leaf tissue, the physiological status of the plants was altered by light starvation and Zn deficiency in attempts to reduce the endogenous precursor supply.

The niacin content of leaves of plants kept in the darkroom for periods from 36 to 96 hours decreased by from 10 to 30 % on the basis of niacin per disk or per gram fresh weight, as compared with the niacin content of similar plants sampled at the start of the dark treatment. The dry weight of the leaf tissue also decreased to the extent that no consistent drop in niacin content appeared when computed on a dry weight basis. In this connection Gustafson (5) has recently reported increases in niacin content of tomato plants with increase in day length at temperatures from 14 to 26° C.

To eliminate the influence of materials stored in other parts of the plant on the niacin metabolism of the leaves, leaf disks were kept in the dark for 24 hours at room temperature. Consistent with the results obtained using intact plants, the leaf disks showed a decrease of niacin content on a per disk or fresh weight basis, compared with controls from the same group of disks frozen immediately after cutting, as is shown in table II.

Leaf disks taken from starved plants or disks kept in the dark for 24 hours were markedly impaired in their ability to synthesize niacin when exposed to light, as is also shown in table II. Infiltration of light-starved disks with the above mentioned precursors, under a variety of conditions of concentration and pH, did not stimulate niacin synthesis in light or dark. Infiltration and incubation of light-starved disks with 1 % sucrose also did not increase synthesis in light or dark.

In contrast to the above observations, leaf disks stored at 5° C in the dark for 24 hours accumulated niacin when subsequently exposed to light at the same rate as disks incubated immediately after cutting.

Since Tsui (12) has shown that the tryptophan content of zinc deficient plants is reduced to a level such that auxin content is also reduced, the niacin content and synthetic potential of leaves from Zn deficient plants was investigated.

Zinc deficient plants were grown as described by Tsui, with mineral nutrients purified according to the method of Stout and Arnon (10). The deficiency symptoms observed were essentially the same as described by Tsui. The tryptophan content of the leaves from deficient plants was reduced in comparison to controls with complete nutrient supply when determined both by Lugg's method as modified by Tsui and by the microbiological method of Greene and Black as modified by Nason (9) with the exception that a vacuum desiccator was not used. The auxin content of the leaves from deficient plants was lower as determined by Avena assay.

The niacin content of the leaves of Zn deficient plants was not lower than that of the controls, and in a series of plants grown during the summer the niacin content of deficient leaves was actually 10 to 20 % higher on a dry weight basis than that of the controls when the first symptoms of deficiency occurred (appearance of excessive anthocyanin and shorter internodes). In a series of plants grown dur-

TABLE I

CHANGES IN AUXIN CONTENT OF LEAF DISKS INCUBATED WITH TRYPTOPHAN FOR 3.5 HOURS AT ROOM TEMPERATURE

CONC. OF DL-TRYPTOPHAN M/LITER	INCUBATION CONDITION	AUXIN CONTENT DEGREES CURVATURE *
0	Unincubated	5.0 ± 1.6
0	Light	3.6 ± 1.1
0	Dark	1.3 ± 1.0
5×10^{-4}	Light (boil)	5.6 ± 2.0
5×10^{-4}	Light	17.0 ± 2.2
5×10^{-4}	Dark	16.9 ± 2.4

* Average from 12 coleoptiles with standard deviation for single extract.

TABLE II

THE EFFECT OF PREINCUBATION OF LEAF DISKS IN THE DARK AT ROOM TEMPERATURE ON NIACIN CONTENT, AND ABILITY TO SYNTHESIZE NIACIN ON SUBSEQUENT EXPOSURE TO LIGHT

EXP.	TIME IN DARK	INITIAL NIACIN CONTENT		NIACIN SYNTHESIS * AS % OF INITIAL NIACIN
		µG/DISK	µG/GM DRY WT.	
1	0	0.140	43.6	+21
	24 hr.	0.126	45.1	+ 7
2	0	0.161	48.9	+25
	24 hr.	0.127	43.8	+ 2
3	0	0.113	43.5	+32
	24 hr.	0.081	38.2	+13
4	0	0.142	44.4	+23
	24 hr.	0.098	37.8	+13

* Niacin increase on dry weight basis after incubation of treated disks in light, experiments 1 and 2 for 4 hours, experiments 3 and 4 for 5 hours.

ing the winter the niacin content of leaves of Zn deficient and control plants was essentially the same. A similar situation had been observed previously by Gustafson (6).

To determine the effect of Zn deficiency on niacin synthesis, disks were cut from the second to fifth leaves (counting from the apex) of plants showing definite deficiency symptoms, but before extensive necrosis of the leaves was apparent. Disks from similar leaves of plants grown on complete nutrient supply served for comparison. In six experiments Zn deficiency did not cause a significant decrease in the ability of leaf disks to accumulate niacin in light.

Incubation of Zn deficient leaf disks with DL- or L-tryptophan did not stimulate niacin synthesis, and in four experiments out of seven caused almost complete inhibition. Unfortunately this inhibition could not be achieved consistently and its significance could not be evaluated on the basis of data available.

It is evident that tryptophan and 3-hydroxyanthranilic acid are not limiting niacin synthesis under any of the conditions described. There is however a limitation of unknown nature imposed on light activated accumulation when leaf tissue is preincubated in the dark at room temperature. In connection with these observations it is noteworthy that in all instances where there is evidence that tryptophan acts as a niacin precursor light is not involved in the conversion. This is especially significant with respect to the observation by Gustafson (5) that the effect of tryptophan in increasing the nicotinic acid content of detached tomato leaves is not affected by light. On the other hand whenever light has been shown to be a factor in niacin synthesis, it has not been possible to show any effect by tryptophan. Thus Terroine (11) was unable to show an effect of tryptophan on light activated accumulation of niacin in excised bean embryos; and Zeijlemaker (15) reports no effect of tryptophan on the nicotinic acid synthesis of pea plants in which light is involved. It would thus appear possible that the light controlled process of niacin synthesis of certain green plants indicates an alternative synthetic pathway in which tryptophan is not involved.

The author is especially grateful to Professor F. G. Gustafson for his encouragement in this investigation.

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ASSAY OF CYTOCHROME OXIDASE ACTIVITY OF SUNFLOWER LEAF TISSUE IN RELATION TO pH VALUE AND CATION CONCENTRATION OF THE BUFFER[1,2]

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Cytochrome oxidase probably occurs in the tissues of most plants and mediates a major portion of terminal respiration. Webster (11, 12) recently demonstrated the presence of a cytochrome oxidase in the tissues of 54 plant species, representing 23 families of dicotyledonous plants. Investigations in the plant physiology laboratory at Rutgers University have indicated the presence of a cytochrome oxidase in sunflower, tomato, soybean, and wheat leaf, and in dodder (*Cuscuta subinclusa*) tissues.

There is evidence that a low or negligible cytochrome oxidase activity reported by some investigators for a number of plant species is at least partly due to the use of buffers at a molarity and a pH which are not optimum for the activity of this enzyme. It has been well established by Howell and Sumner (6) for urease, by Massey (7) for fumarase, and by Gilbert and Swallow (5) for Q-enzyme that the pH optimum for maximum activity is dependent upon ion concentration of the reaction mixture, and that definition of the pH optimum or ion concentration alone does not satisfactorily characterize an enzyme reaction. Smith and Stotz (9) reported that the rate of oxidation of ferrocytochrome c by rat liver, heart, kidney, and intestine homogenates increased slightly from pH 6.5 to 7.0. Bertrand and Gavard (1) found the optimum pH of beef heart cytochrome oxidase to be 8.0. Quinlan-Watson and Dewey (8) reported a pH optimum of 7.1 to 7.3 for various animal tissues. Ducet and Rosenberg (4) reported a pH optimum of 7.4 and a phosphate buffer concentration of about 0.05 M for potato leaf particulate material. Cooperstein, Eichel, and Wainio (2) found the optimum for beef heart cytochrome oxidase to be about 7.0. In a later study, Wainio et al (10) reported that the rate of oxidation of ferrocytochrome c is markedly influenced by the molar concentration of the Na_2HPO_4-KH_2PO_4 buffer used. Maximum activity of the beef heart cytochrome c–cytochrome oxidase system was found to increase and the optimum molarity of the buffer to decrease as the pH was changed from 7.4 to 6.2. Wainio and his coworkers in more recent, unpublished work, have demonstrated that desoxycholate preparations of beef heart cytochrome oxidase assayed spectrophotometrically show maximum activity in a phosphate buffer of pH 6.0 with a cation concentration of 0.112 M. They found that the activity of the enzyme is not related to total ionic strength or to the anion concentration of the buffer.

An investigation was carried out to determine the optimum pH and cation concentration at which the cytochrome oxidase of sunflower leaf tissues exhibited its maximum activity. The plants used (*Helianthus annuus* L.) were grown in culture solutions containing adequate levels of the essential inorganic nutrients. A pH optimum curve was determined by utilizing a series of 0.1 M phosphate buffers (Na_2HPO_4–KH_2PO_4), ranging in pH from 5.0 to 7.0. The optimum cation concentration was determined by using a series of phosphate buffers with cation concentrations ranging from 0.019 to 0.160 M, each at pH 6.2. Cytochrome oxidase of whole sunflower leaf homogenates was assayed spectrophotometrically by the method of Wainio et al (10) and Darby and Goddard (3). A similar method was also used by Webster (11). The leaf homogenate was prepared by grinding 1 gm of young leaf tissue in a cold mortar and pestle. Maceration was completed in a cold Ten Brock glass homogenizer. The homogenate was then diluted to 25 ml with cold, deionized water. Aliquots of 0.1 ml were used for activity determinations. Figure 1 indicates, in terms of the velocity constant (K sec^{-1}), that the pH optimum for the sunflower cytochrome c–cytochrome oxidase reaction is 6.0, and figure 2 indicates that the cation concentration optimum at pH 6.2 is about 0.130 M.

These values agree reasonably well with those found for animal tissues by Wainio and his coworkers. They do not agree, however, in all respects with the results of Ducet and Rosenberg (4). Whereas their cation concentration was not stated di-

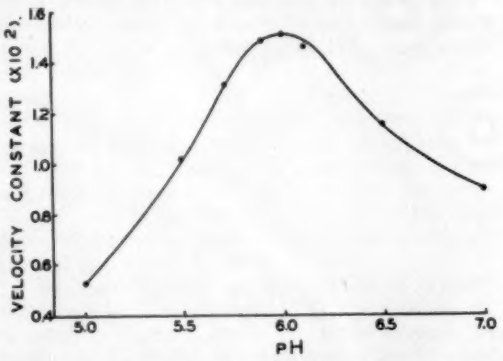

FIG. 1. Effect of variations in pH of phosphate buffer solutions on the cytochrome oxidase activity of sunflower leaf tissues. Values are expressed as the velocity constant for a first-order reaction (K sec^{-1}).

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[2] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Plant Physiology and Bureau of Biological Research, New Brunswick.

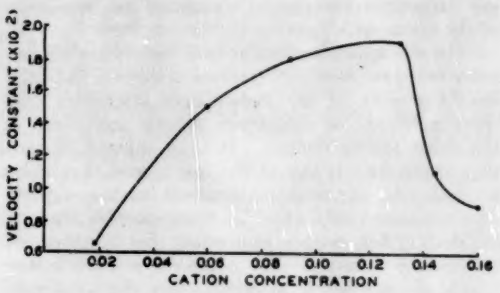

FIG. 2. Effect of variations in cation concentration of phosphate buffer solutions of pH 6.2 on the cytochrome oxidase activity of sunflower leaf tissues. Values are expressed as the velocity constant for a first-order reaction (K sec^{-1}).

rectly, the optimum buffer concentration was approximately 0.05 M phosphate which would correspond to a cation concentration of about 0.1 M. This concentration falls in the vicinity of the optimum cation concentration found by us. However, there is some difference in pH optima found which may be due to their use of a manometric instead of a spectrophotometric method.

It appears from the work reported that a combination of a pH of about 6.0 and a cation concentration of the phosphate buffer of about 0.130 M is probably the most favorable one for the determination of cytochrome oxidase activity in plant tissues by the spectrophotometric method employed.

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THE EFFECT OF CARBON MONOXIDE ON RESPIRATION IN HIGHER PLANTS[1]

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Current studies (1, 2, 5) have indicated that a cytochrome oxidase is found in most of the tissues of higher plants. The widespread distribution of this enzyme has raised the possibility of its general participation in plant respiration in a manner similar to that in animals, yeast, and aerobic bacteria. Unquestionable proof of the participation of cytochrome oxidase in plant respiration is very difficult, but the relative importance of cytochrome oxidase and tyrosinase (or other copper containing enzymes) in respiration may be estimated by use of the carbon monoxide inhibition techniques of Warburg (4). It is well established (3, 4, 5) that the carbon monoxide inhibition of cytochrome oxidase activity is eliminated by light while the carbon monoxide inhibition of tyrosinase is insensitive to light. This difference in properties provides a means of differentiating between the activities of these oxidases in plant respiration.

In connection with some previously reported studies (5) on the occurrence of cytochrome oxidase in plants, the author had occasion to examine the effect of carbon monoxide and light on the respiration of a number of plant tissues. The respiration of root (1 cm sections) or other tissue (0.5 mm slices) in 0.05 M potassium phosphate buffer (pH 7.1) was measured at 25° C by standard manometric tech-

[1] Received March 15, 1954.

niques in various ratios of carbon monoxide or nitrogen to oxygen. The results reported here were obtained with 95 % CO and 5 % O_2. As is evident from table I, the respiration of all eleven tissues studied was strongly inhibited by carbon monoxide. In ten of the eleven cases examined the inhibition was largely or completely eliminated by irradiation of the tissue with light.

TABLE I

EFFECT OF CARBON MONOXIDE AND LIGHT ON THE RESPIRATION OF NON-PHOTOSYNTHETIC TISSUES OF SOME HIGHER PLANTS

PLANT AND TISSUE	PERCENT INHIBITION OF RESPIRATION WITH 95 % CO AND 5 % O_2*	
	In the dark	*In the light* **
Fagopyrum esculentum L. (buckwheat)—root	65	3
Delphinium Ajacis L. (larkspur)—root	51	6
Raphanus sativus L. (radish)—root	38	19
Pyrus Malus L. (apple)—fruit	45	45
Beta vulgaris L. (beet)—root	43	9
Phaseolus vulgaris L. (bean)—root	61	6
Phaseolus limensis L. (lima bean)—root	63	0
Medicago pratense L. (alfalfa)—root	56	0
Nicotiana Tabacum L. (tobacco)—root	43	7
Cucumis sativus L. (cucumber)—fruit	54	10
Helianthus annuus L. (sunflower)—root	55	0

* Percent inhibition as compared with a 95 % N_2 and 5 % O_2 control.

** White light of about 300 fc incident on the vessel.

The one exception, apple fruit tissue, has consistently failed to show any reversal of carbon monoxide inhibition even at far higher light intensities than those necessary to completely reverse inhibitions in the other tissues studied. It is of interest to note that apple fruit is one of the few tissues that failed to show any significant cytochrome oxidase activity in a previous study (5). It seems possible that we are dealing here with a respiration that is either not cytochrome linked or that is altered in such a way that it is not possible to demonstrate the occurrence of the cytochrome oxidase.

Despite the results with apple, the far more important fact remains that in ten of the eleven tissues examined the evidence fairly well precludes the participation of a tyrosinase and definitely supports the participation of a cytochrome oxidase in respiration. The results obtained here, therefore, provide further evidence for the general importance of cytochrome oxidase in plant respiration.

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NOMENCLATURE OF CHEMICAL PLANT REGULATORS—A CRITICISM

In the May, 1954, issue of Plant Physiology there appears a report of a committee of the American Society of Plant Physiologists on the nomenclature of Chemical Plant Regulators. The undersigned was a member of that committee, but did not sign that report, as he was opposed to various points in the set of definitions proposed by the majority of the committee.

REGULATOR: The word Regulator has a status in Biology as a term for a substance by which living organisms maintain the harmony and balance of their various physiological processes in spite of the action of external factors. To regulate, according to Webster's Dictionary, means "to adjust so as to work accurately and regularly." 2,4-D and several other synthetic growth substances do not do this. 2,4-D, therefore, is not a regulator; and REGULATOR should not be used as a broad term including substances, influencing a variety of physiological processes, another term is needed, ERGON might serve this purpose; but within the field of growth processes the term GROWTH SUBSTANCE is sufficient.—Further, the assignment of the Committee is "to consider and propose a uniform nomenclature on growth substances."

The Minority, therefore, proposes the following set of definitions.

PROPOSED DEFINITIONS

GROWTH SUBSTANCES are organic compounds which at low concentrations promote, inhibit, or qualitatively modify growth. Their effect does not depend on their caloric value or their content of essential elements.

Comment: On account of the ambiguity of the word "nutrient" (cf. proposal by the Majority), particularly when both autotrophic and heterotrophic organisms are considered, this term should be avoided, and is unnecessary.

GROWTH HORMONES are growth substances which regulate growth and are produced by the organism itself. They usually move within the organism from a site of production to a site of action.

Comment: For the meaning of "regulate," see above.

AUXINS are growth substances characterized by their capacity to induce elongation in shoot cells, when applied at suitable concentrations.

Auxins may, and generally do, affect other processes besides elongation, but the effect on elongation is considered critical. Auxins are generally acids with an unsaturated, cyclic nucleus or their derivatives. Indole-3-acetic acid is commonly used as a standard in determining auxin activity.

Comment: The Minority is opposed to the definition of a group by one of its members and to the statement that auxins "resemble" indole-3-acetic acid in physiological action.

AUXIN PRECURSORS are compounds which in the plant can be converted into auxins.

ANTI-AUXINS are compounds which inhibit competitively the action of auxins.

An anti-auxin must at some concentration promote the auxin-inhibited elongation in roots.

GROWTH-INHIBITORS are substances which retard cell enlargement in both shoot and root cells and have no stimulatory range of concentration.

Comment: This definition is necessitated by the preceding, specific definition of anti-auxins. A logical, systematic name for oligodynamic growth inhibitors would be auxocholins, analogous to Kockemann's blastocholins.

WOUND HORMONES are growth substances which induce cell divisions and are liberated upon injury to tissue.

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BIOCHEMICAL PREPARATIONS.—Vol. I ($3.00), 76 pages. H. E. Carter, Editor. Vol. II ($3.00), 109 pages. E. G. Ball, Editor. Vol. III ($3.50), 128 pages. E. E. Snell, Editor. John Wiley and Sons, Inc., 440 Fourth Avenue, New York, New York.

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The introduction to each preparation contains a concise outline of the method to be used along with the equations of the reaction, which are presented using structural formulae to indicate clearly the modifications made in the starting material to obtain the desired end-product. Each method has at the end a conscientious bibliography of other proposed techniques for preparing the same compound. In addition, the front of each volume contains a running index to the syntheses of biologically important compounds which have appeared in the parent publication, *Organic Syntheses*.—J. A. SCHIFF.

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TABLE OF CONTENTS

A Porphyrin Pigment from Photosensitive Nec[rotic] Tissues. W. Tedd and A. W. Galston ..... Green 311

Position of C[14] in Alcohol and Carbon Dioxide Produced from Corn Root Tips. Harry Beevers and Melvin [illegible] ..... [Glucose by] 318

The Direct Oxidation Pathway in Plant Respiration. [illegible] ..... [Martin] Gibbs 322

Investigation into the Function of Pyridoxin as a Growth [illegible] ..... [Grown] Tomato Roots. William G. Boll 325

Studies on the Chemistry of the Living Bark of the Black Locust [illegible] ..... [Relation] to Its Frost Hardiness. *VII. A Possible Direct Effect of Starch* [illegible] ..... [Susceptibility] of Plants to Freezing Injury. D. Steinhoff and D. [illegible] 331

The Availability of Magnesium from Organic Carriers. [illegible] ..... [Substrates.] Stuart Dunn and Suzanne S. Roberts 337

Translocation of Radioactive Isotopes from Various Regions [illegible] ..... [Roots of Barley] Seedlings. Herman H. Weber and Paul J. Kramer 342

Effect of Differential Nitrogen, Potassium, and Magnesium [illegible] ..... [Valencia] Orange Trees in Sand Culture on Mineral Composition [illegible] ..... [Leaves] and Fibrous Roots. Paul F. Smith, Walter [illegible] ..... [Reuther,] and Gustave Hrdlicka 349

The Influence of Mineral Nutrition on the Organic [illegible] ..... [Hydroperi]cum esculentum. Apolinario R. Carangal, [illegible] ..... [Warner,] and R. C. Burrell 355

Nitrogenous Constituents of Sap Exuded from the Sapwood of [illegible] ..... [Saccharum.] John K. Pollard, Jr., and Thomas Sproston 360

Studies in Plant Metabolism. IV. Comparative Effects of 2,4-Di [illegible] ..... [Phenoxyacetic] Acid and Other Plant Growth Regulators on Phosphorus [illegible] ..... [in Bean] Plants. S. C. Fang and Joseph S. Butts 365

Promotion by Zinc of the Formation of Cytochromes in Un [illegible] ..... [Nitrogens.] Phyllis Wenkel Grimm and Paul J. Allen 369

Auxin Physiology in Bean Leaf Stalks. Koan Sifton and [illegible] 377

Incorporation of Radioactive Glutamic Acid into the Proteins of Higher Plants. George C. Webster 382

The Effect of Temperature on the Behavior of Malic Acid and [illegible] ..... [Leaves of] *Bryophyllum calycinum Cultured in Darkness.* Hubert [illegible] 385

Brief Papers:

Smog Injury and Root Infection. C. E. Yarwood and [illegible] 393

Precursor Relationships in Light Activated Accumulation of [illegible] ..... [Leaf Disks] *from Normal, Light-starved, and Zinc Deficient Tomato Plants.* [illegible] L. Crane 396

Assay of Cytochrome Oxidase Activity of Sunflower Leaf Tissue [illegible] ..... [pH] *Value and Cation Concentration of the Buffer.* Leonard [illegible] ..... [Rist,] W. Rex Robbins, and Walter W. Waino 398

The Effect of Carbon Monoxide on Respiration in Higher [illegible] ..... [William C.] Webster 399

Nomenclature of Chemical Plant Regulators—A Criticism. Pool [illegible] 400

Book Reviews. J. A. Schiff 401